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REDIRECTING DENDRITIC CELLS AND MACROPHAGES TOWARDS TUMOR REJECTION

by
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A thesis submitted to the Open University of London for the degree of Doctor
of Philosophy in Life and Biomolecular Sciences

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ABSTRACT

The liver expressed chemokine CCL16/LEC exerts chemotactic activity on human monocytes and lymphocytes and is also active on murine cells. An adenovirus encoding the chemokine CCL16 was used to test whether this chemokine might inhibit pre-existing tumors in mice. AdCCL16, but not control empty vector, when injected in established TSA mammary carcinoma, significantly delayed tumor growth. Immunohistochemistry revealed accumulation at tumor site of CD4⁺ and CD8⁺ T cells, macrophages, and dendritic cells (DC) the latter being also enriched in draining lymph nodes. Despite the robust and rapid immune response triggered by intratumoral injection of AdCCL16, the lesions were not completely rejected. However the same treatment given before surgical excision of primary lesions prevented metastatic spread and cured 63% of mice bearing the 4T1 mammary adenocarcinoma. The finding of an hostile tumor microenvironment preventing innate immunity explains the weak effect on the primary tumor. As CCL16 promotes accumulation of macrophages and DC at the site of pre-established tumor nodules this treatment was combined with the TLR9 ligand CpG and with anti-interleukin 10-receptor (IL10R) antibody to contrast the local tumor-induced immunosuppression. CpG plus anti-IL10R promptly switched M2 infiltrating macrophages to M1 that, triggering a strong innate response, debulked large tumors within 16 hrs. Tumor infiltrating DC matured and migrated in parallel with the onset of the innate response, allowing the initiation of adaptive immunity before the diffuse hemorrhagic necrosis halted the communication between tumor and draining lymph node. Treatment of B6>CXB6 chimeras implanted with BALB/c tumors induced an efficient innate response but not CTL-mediated tumor lysis; in these mice tumor rejection did not exceed 25%. The requirement of CD4 help for an effective CTL induction was shown in CD40KO, as well as in mice depleted for CD4 T cells during the priming rather than the effector phase. Together the data describe the critical requirements for the immunological rejection of large tumors: a hemorrhagic necrosis initiated by activated M1 macrophages and a concomitant DC-migration to DLN for subsequent CTL priming and clearing of any tumor remnants.

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ABBREVIATIONS

Ab	antibody
APC	antigen presenting cells
BCG-CWS	bacillus Calmette-Guérin cell-wall skeleton
BM-DC	bone marrow derived dendritic cells
bp	base pair
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD40L	CD40 ligand
cDNA	complementary DNA
CEBP	CCAAT/enhancer binding protein
chTNT-3	human tumor necrosis treatment antibody
CpG-ODN	CpG oligodeoxineucleotide
CREB	cAMP response element-binding protein
CSF	colony stimulating factor
CTL	cytotoxic T cells
CXCL	CXC chemokine ligand
DC	dendritic cell
DLN	draining lymph nodes
DNA	deoxyribonucleic acid
E:T	effector target cell ratio
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
FACS	fluorescence-activated cell sorter

FCS	foetal calf serum
FITC	fluorescein
GKO	Interferon- γ KO mice
GM-CSF	Granulocyte/macrophage colony stimulation factor
h	hour(s)
i.p.	intraperitoneal
i.v.	intravenous
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
ITR	inverted terminal repeat
kb	kilobase
kDa	kilodalton
KO	knock out
LN	lymph nodes
LPS	lipopolysaccharide
m	monoclonal
mAb	monoclonal antibody
MCMV	mouse cytomegalovirus
MHC	major histocompatibility complex
MMTV	mouse mammary tumor virus
MOI	multiplicity of infection
mRNA	messenger RNA
MuLV	moloney leukemia virus
MyD88	myeloid differentiation primary response gene (88)
NF κ B	nuclear factor kappa B

NK	natural killer
NO	nitric oxide
OVA	ovalbumin
PAMP	pathogens-associated molecular pattern
PBS	phosphate buffered saline
PDC	plasmacytoid DC
Pfu	plaque forming unit
PMN	polymorphonuclear
r	recombinant
RIP-Tag	rat insulin promoter (RIP)- T Ag (Tag) tumor model
RNA	ribonucleic acid
rpm	revolution per minute
s.c.	subcutaneous
SD	standard deviation
SLE	systemic lupus erytematosus
STAT	signal-transducer and activator of transcription
[³ H]TdR	thymidine deoxyribose
TAA	tumor associated antigen
TAM	tumor infiltrating macrophages
TAM	tumor associated macrophages
TAP	transports associated with antigen processing
TCR	T cell receptor
TGF	transforming growth factor
TGF	transforming growth factor
Th	T helper
TIDC	tumor infiltrating DC

TIR	Toll/interleukin-1 receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAIL	TNF related inducing ligand
UC	ulcerative colitis
VEGF	vascular endothelial growth factor
Vol	volume

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CHAPTER 1 INTRODUCTION

1.1 Immunotherapy of cancer

The immune system has evolved strategies to efficiently localize and destroy disease-inducing agents. The idea that the same defence mechanisms might be directed against cancer started the age of tumor immunology.

The dramatic advances in understanding the basic aspect of the immune system as well as the progress in genetic engineering techniques highlighted the possibility that the immune system could be manipulated to destroy cancer, starting the era of immunotherapy. The following sections of this introduction describe the newest approaches in cancer immunotherapy. Evidence for tumor-induced suppression of the patient's immune system has always existed; a section of this introduction will be devoted to immune escape mechanisms and how these mechanisms can be manipulated for therapy of established malignancies.

1.1.1 Differences between therapy and prevention of cancer

Immunotherapy approaches can be divided in two main categories: i) those targeting healthy individuals with a high risk of developing cancer or individuals with preneoplastic lesions, ii) those that aim at curing established tumors.

Immunoprevention of cancer has many theoretical advantages. Individuals in which the immune system has not been yet negatively instructed by tumor are expected to mount a more efficient immune response.

In this setting two treatments can be foreseen: enhancement of non-specific immunity and specific vaccination with tumor associated antigens (TAAs) [reviewed in ref. (Forni et al., 2000; Lollini and Forni, 1999)].

Numerous data obtained in cancer patients as well in mouse models of cancer have shown that proinflammatory cytokines such as IL-2, IL-12 and interferons elicit modulation of natural immune reactivity that can results in antitumor response.

In particular, non-specific stimulation using IL-12 has been extensively tested in animal cancer models. BALB/c female expressing the MMTV-driven rat oncogene HER2/neu, develop hyperplasia starting at 5 weeks of age, which then progresses to in situ carcinoma and at 33 weeks of age all mammary glands display large tumors.

Chronic administration of low doses of IL-12, starting at 1 weeks of age till the 15th week, markedly delays the progression of mammary carcinogenesis by interfering with the passage from atypical hyperplasia to invasive carcinoma, suggesting an indirect inhibition of the angiogenic switch. The same treatment, started at the stage of invasive carcinoma, loses its effectiveness posing major concern on the use of non-specific therapy to treat already established lesions (Boggio et al., 1998).

Similarly, IL-12 triggered mechanisms inhibited chemical induced carcinogenesis. BALB/c mice injected subcutaneously with 3-methylcholantrene showed reduced tumor appearance and reduced tumor incidence when treated chronically with IL-12 (Noguchi et al., 1996).

Specific vaccination of persons at risk or with preneoplastic lesions represents a completely different scenario. In this setting the identification of altered gene products predictably destined to become a TAA is the first step towards the engineering of effective vaccines (Forni et al., 2000). The protection provided by vaccination of BALB/NeuT mice, with a plasmid containing the extracellular domain of the *neu* oncogene, suggests that prophylactic vaccination may be effective (Rovero et al., 2000) (Spadaro et al., 2004).

The preventive effect of early vaccination is markedly increased by combination with IL-12 treatment. In addition to improving the protection rate of the DNA vaccine, IL-12

significantly delayed the initiation of antigen-specific anti-tumor vaccination (Spadaro et al., 2004).

Numerous studies on animal models suggest that immunoprevention is effective in inhibiting tumor onset, but many issues limit the translation of this approach to the clinic. First there are very few situations in which genetic screening can select a population with a specific risk of cancer where prevention could be contemplated. Second, one should be absolutely certain that preventive vaccination would not elicit any autoimmune effect considering that many TAA are inappropriately expressed self-antigens.

A more realistic approach that follows true clinical need considers the treatment at early diagnosis or post-surgery. This approach is centered on manipulation of the immune system to reject rather than prevent tumors.

The final goal of therapy of established tumors is development of T cell and/or Ab-responses against TAA that are able to destroy tumor and generate long lasting immunity that will protect against minimal residual disease and relapse.

The most recent therapeutic attempts use DC that have been ex-vivo manipulated or stimulated in vivo, to present tumor antigens. Other attempts aim at stimulating the innate response as a preliminary step towards adaptive response and subsequent memory activation (see next sections for further details).

1.2 DC in cancer immunotherapy

1.2.1 Dendritic cell as antigen presenting cells

Dendritic cells belong to a family of antigen presenting cells that orchestrate signals derived from different component of the immune system. At the immature state, DC reside in peripheral tissues and act as sentinel sensing antigenic environment for the presence of pathogens and any danger signals.

The encounter with pathogens or with a highly inflamed tissue initiates a series of events that culminates with antigen presentation to T cells residing in lymphoid tissues [reviewed in ref. (Banchereau and Steinman, 1998)].

The kind of immune response that is produced by DC depends on how antigens are presented to T cells.

Antigenic stimulation needs to be coupled with an inflammatory or danger signal to fully activate DC maturation, transforming a cell equipped to capture antigens into a cell equipped to process and present them to T cells. DC are indeed provided with receptors belonging to the toll-like receptor (TLR) family that recognize pathogens and whose triggering initiate the process of maturation. Other cell types such as macrophages also express TLR that upon triggering induce the production of inflammatory cytokines (TNF- α , IL1- β , IL-12, IFN- γ) that are instrumental for proper phagocytosis and activation of DC (Banchereau and Steinman, 1998).

Antigens from pathogens infecting directly APC (called endogenous antigens) are transported to the endoplasmatic reticulum (ER) by a specialized protein complex named TAP (transported associated with antigen presentation) and here loaded into the groove of MHC class I molecules. The peptide-MHC-I complexes are finally conveyed to the cell surface via the secretory pathway. Once DC reach lymphoid tissues the processed antigens are presented to CD8 T cells that are able to kill directly the infected cells. Extracellular pathogens, on the contrary, are engulfed by DC and degraded in phagosomes (membrane-bound cellular compartments); here the produced peptide fragments are loaded onto MHC class II molecules that once translocated to the cell surface stimulates CD4 T helper cells. Helper T cells are not able to kill directly the pathogens instead they trigger the production of antigen specific antibodies and produce inflammatory stimuli that contribute to eliminate pathogens (Banchereau and Steinman, 1998).

The rules governing antigen presentation are even more complex, in fact DC can present exogenous antigens also via the MHC class I pathway to CD8 T cells, a process named cross-presentation. This pathway is important to generate specific CTL immunity against pathogens that do not infect DC. If direct presentation was the only way to elicit CTL response, pathogens would escape immune response by avoiding DC infection. Instead DC can capture and cross-present pathogen antigens from other infected cells avoiding such escape.

Several types of antigens have been reported to be cross-presented. Among them soluble proteins, intracellular bacteria, parasite and cellular antigens.

In particular cross-presentation of cellular antigens is thought to be important to generate CTL response against tumor cells or self-tolerance against tissue-derived antigens [reviewed in ref. (Heath et al., 2004; Melief, 2003)]. Indication that cellular antigens can be captured and indirectly presented by APCs can be found in early experiments investigating the MHC-restriction of responses to minor histocompatibility antigens. Bevan *et al*, primed (BALB/c X BALB/B) F1 hybrids mice (H-2^d X H-2^b) with cells from C57BL/10 (B10) mice that share the H-2^b MHC molecules but diverge in their expression of B10 minors. These authors found that not only the H-2^b-restricted CTLs specific for B10 minors were induced after immunization, but H-2^d-restricted CTLs were found as well. To induce the H-2^d-restricted response, minor antigens must have been transferred to APCs of host origin, Bevan named this process “cross-priming” referring specifically to CTL associated with presentation of cellular antigens captured and presented by APC of the host-BM compartment (Bevan, 1976).

In the past years many studies have investigated the processes governing cross-presentation. The clarification of such process is essential especially if considering that cell-associated antigens has been shown to promote a superior CD8 T cell response *in vivo* than soluble antigens raising the possibility that immunotherapeutic strategies

implementing cross presentation of tumor associate antigens would results in a more vigorous CTL response (Li et al., 2001).

The existence of a fusion process between the phagosomes and the ER has been reported, by three different groups, as essential for extracellular antigens to escape the phagosomes and to be loaded onto MHC class I molecules (Ackerman et al., 2003; Houde et al., 2003) (Guermónprez et al., 2003). The phagocytated antigens are then transported to the cytosol by a protein complex named Sec 61. Once the exogenous proteins have reached the cytosol they are degraded by the proteasome and resulting peptides are translocated by TAP into the lumen of the same phagosomes, before loading on phagosomal MHC class I molecules. Therefore, cross-presentation in dendritic cells occurs in a specialized, self-sufficient, ER-phagosome mixed compartments demonstrating that the phagosome is a fully competent antigen processing compartments for the MHC class I pathway.

Norbury *et al.* demonstrated that cells expressing stable proteins were capable of cross-priming, whereas those expressing minimal peptide were not, indicating that cross priming occurs when intact proteins are transferred from donor cells to DC (Norbury et al., 2004).

Moreover, the location of the antigenic peptide within the protein is an important factor limiting the efficiency of cross-presentation. Indeed, Wolkers *et al.* constructed a chimeric secretable GFP protein in which the antigenic epitope was placed at the N terminus within a signal sequence or near the C terminus. Only the protein in which the epitope was at the C-terminus could be cross-presented revealing the importance of location and an unexpected bias of epitopes located within functional signal sequences. These and other studies indicated that cellular proteins rather than peptides are the source materials for cross presentation, suggesting that strategies aimed at maximazing steady state expression of the target antigen and its stability within the donor cells would be predicted to generate a better CTL response (Heath et al., 2004; Melief, 2003).

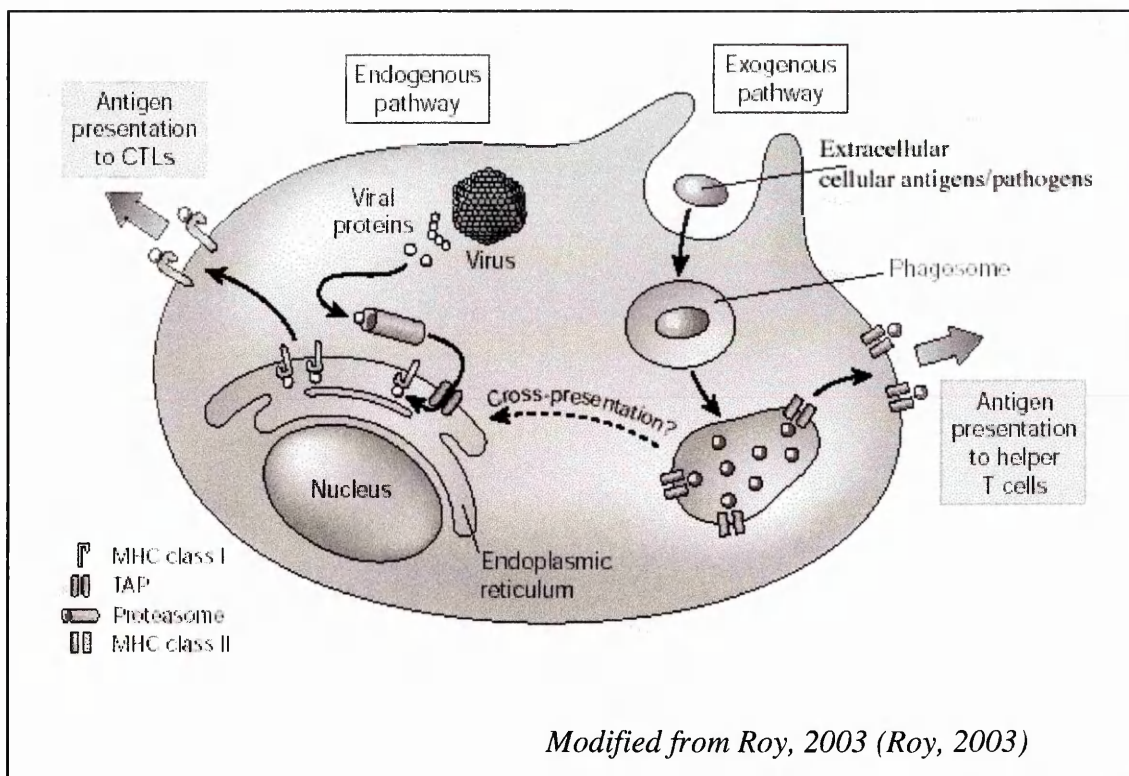


Figure 1.1 DC process intracellular or extracellular antigens differently

It has been argued that cross priming of TAA by DC is an inefficient and rare process and therefore not able to elicit protective anti-tumor immunity [reviewed in ref. (Ochsenbein et al., 2001; Zinkernagel, 2002)]. The idea here is that tumor cells need to migrate to draining lymph nodes to directly present their own antigens in secondary lymphoid organs. In the cases where tumor cells metastasize to draining lymph nodes authors found immune activation based on a direct presentation of TAA to CD8 T cell. Therefore, it has been proposed that T cell tolerance to tumors is simply the consequence of an immunological ignorance due to the fact that most tumors never reach secondary lymphoid organs.

However, virtually, all the studies investigating this phenomenon, report that the predominant pathway of presentation of TAA to CD8 T cells is the cross-presentation pathway by host APC.

The reason for the inefficiency of this process resides on the findings that tumor infiltrating DC are insufficiently activated and therefore incapable of efficiently cross-priming TAA

(Melief, 2003). Indeed, if tumor bearing mice were treated with anti-CD40 agonist Ab, a DC activation signal, a strong expansion of CD8 tumor-specific T cells was obtained in peripheral lymphoid tissues (van Mierlo et al., 2002). The same study demonstrated that CD11c⁺ DC in DLN, rather than directly tumor cells, present TAA to CD8 T cells. Such phenomenon was strictly dependent on the TAP pathway reinforcing the idea of an indirect DC-mediated cross-presentation of TAA (Melief, 2003).

Pardoll's group has demonstrated that cross-presentation is implicated in the induction of CTL against tumors. Indeed, CTL against a tumor cell line transfected with the gene of the influenza nucleoprotein (NP) were restricted to the haplotype of the host BM compartment (Huang et al., 1994a).

Tumor antigens are thought to enter into the cross-presentation pathway after undergoing cell death (Heath et al., 2004; Melief, 2003). Two types of cell death have been described: apoptosis and necrosis. The main difference between (programmed) apoptosis and accidental/ toxic (necrotic) death is that apoptosis results in the ordered fragmentation of the cells and leads to a rapid phagocytosis by neighbour cells and APC without inducing cell activation or inflammation. Apoptotic cells accumulation in patients affected by SLE or other autoimmune syndromes develop into the late phase of apoptosis, also called secondary necrosis, function as danger signals and activate the immune system.

It was demonstrated that although DC can engulf both necrotic and apoptotic bodies, only phagocytosis of necrotic bodies results in up regulation of co-stimulatory molecules and the capacity to prime T cells. Therefore it was proposed that phagocytosis of apoptotic cells by DC leads to tolerance to self or tumor antigens while necrosis leads to full activation of T cell immunity (Sauter et al., 2000).

1.2.2 Dendritic cell migration and priming of T cells

After antigen exposure in the context of an inflamed site, DC undergo a process of maturation that includes up-regulation of costimulatory molecules, of MHC-class I and II complex and secretion of cytokines such as IL-12, TNF- α , IL-6. Maturation is accompanied by the acquisition of migratory capacity that allows DC to reach the appropriate lymphoid compartment for priming of T cells (Banchereau and Steinman, 1998). Exogenous mediators of DC maturation are the already mentioned Toll-like receptor ligands such as LPS, CpG motifs, double strand RNA and bacterial toxins, all components of pathogenic bacteria and viruses. Endogenous mediators of maturation are cytokines such as TNF- α , IFN- γ , IL-1 β which are released locally in inflamed tissue.

In a very recent work, Sporri and Reis e Sousa (Sporri and Reis e Sousa, 2005), demonstrated the existence of two distinct mature states for DC *in vivo*. These authors generated mixed bone marrow chimeras containing a DC subset able to respond directly to pathogens signalling but expressing the wrong haplotype for presenting an antigen to a test CD4 T cell population; the second subsets of DC have the right haplotype for presentation but are unable to respond directly to the TLR triggering. Inoculation of TLR ligands in these mice (LPS or CpG) elicited similar maturation phenotype by both DC subsets, with the first subsets maturing directly via TLR triggering and the second one via inflammatory cytokines produced as secondary mediators following TLR ligands inoculation. Although CD4 T cells proliferate upon antigen encounter in these chimeras they were not able to develop in effector cells able to produce IFN- γ or IL-4. These experiments demonstrate that DC that did not directly receive TLR signals are unable to develop immunity despite their ability to show a mature phenotype.

Acquisition of migratory capacity is concomitant with the upregulation of adhesion molecules such as CD44, $\alpha_6\beta_1$ integrins and chemokine receptors switching [reviewed in ref. (Cavanagh and Von Andrian, 2002)]. Immature DC express receptors for

inflammatory chemokines such as CCR1, 2, 3, 5 and 6. Upon maturation these are down-regulated while CCR7 becomes readily upregulated. CCR7 has two ligands CCL21 and CCL19; CCL21 is expressed by the endothelium of the lymphatic vessels by the high endothelia venules (HEV) and by the stromal cells of the T cell area, while CCL19 is released by mature DC and stromal cells in the T cell area of lymph nodes and spleens (Cavanagh and Von Andrian, 2002). CCL21 is upregulated on lymphatic endothelial cells after administration of inflammatory stimuli as TNF- α and its up regulation is one of the mechanisms by which inflammation promotes DC entering into the lymphatic vessels (MartIn-Fontecha et al., 2003).

Mature DC entering the draining LN are driven into the paracortical area in response to CCL21 and CCL19 expressed by cells spread over the T cell zone (Cavanagh and Von Andrian, 2002).

Lack of CCR7 or its ligand has profound effects on lymph nodes structure and lymphocyte trafficking. Indeed, both Langherans cells and dermal DC were found unable to migrate to draining lymph nodes in the absence of CCR7 or in presence of blocking antibody to CCL21 (Forster et al., 1999) (Gunn et al., 1999).

In mice that have a spontaneous mutation of CCL19, DC fail to enter in the T cell zone of the lymph nodes (Nakano and Gunn, 2001).

Figure 1.2 describes some of the events following antigen exposure in an inflamed tissue environment.

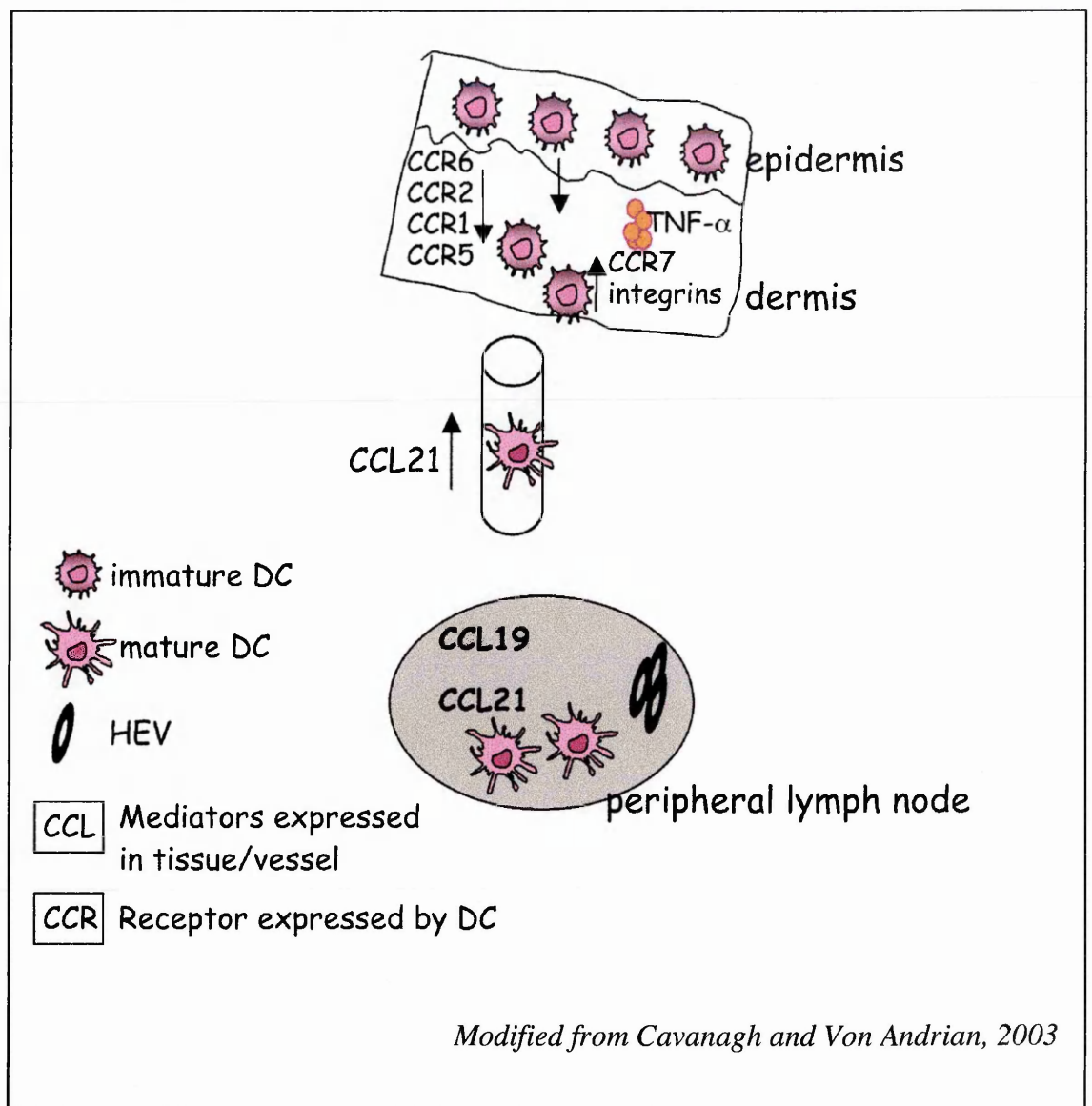


Figure 1.2 Schematic representation of dendritic cell migration from peripheral tissues to draining lymph nodes.

Dendritic cells capture antigens in peripheral tissue and as a consequence of the inflamed environment mature and migrate to peripheral lymphoid tissues. Homing to the LN and localization in the T cell area is orchestrated by chemokines.

Once in the lymph nodes, DC form an immunological synapse with T cells and activate them. Activation occurs when DC display the appropriate ligands to the responding T cells together with the proper engagement of TCR (T cell receptor) on T cells. The strength of the signal depends on different factors: 1) the amount of MHC-peptide molecules that bind to TCR, 2) the level of costimulatory molecules on DC and 3) the duration of the synapse.

Once entered in the first cell division T cells proliferate and become able to respond to IL-2, which is produced by the same proliferating T cells.

The strength of the signal together with the polarizing cytokines present in the environment determines the class of the immune response. CD4 T cells polarize towards T helper 1 (Th1) secreting IFN- γ or Th2 producing IL-4, IL-5 and IL-13. Th1 are adept at macrophage activation and immunoglobulin (Ig) selection for isotypes that mediate Ab-dependent cellular cytotoxicity and complement activation against intracellular pathogens, including viruses, bacteria yeast and protozoa. Th2 cells are involved in the development of humoral immunity protecting against extracellular pathogens. Polarization towards Th1 and Th2 is promoted by IL-12 and IL-4 respectively [reviewed in ref. (Lanzavecchia and Sallusto, 2000; Lanzavecchia and Sallusto, 2001)].

For tumor cell and defined antigen-based therapeutic approaches T cell depletion experiments have demonstrated that both CD4 and CD8 T cell are needed for an optimal response. Because the majority of non-haematological tumors express MHC class I but not MHC class II molecule, it has been proposed that CD4 are necessary for the generation of tumor specific CTL (Hung et al., 1998).

It was proposed that DC have a rather passive relationship with the killer CD8 T cells mainly functioning in stimulating CD4 T helper cells to produce the appropriate cytokines, such as IL-2, that favours the CTL differentiation (Fearon et al., 1990).

However, recent studies suggest a much more dynamic model in which CD4 T cell licences DC to correctly activate CD8 T cells. Indeed, during DC-CD4 T cell cross-talk both cells become reciprocally stimulated [reviewed in ref. (Behrens et al., 2004)].

In 1998, three groups reported independently that CD40 on DC and CD40L on CD4 T cells were the prime means of helper-dependent DC licensing for CTL induction (Ridge et al., 1998) (Bennett et al., 1998) (Schoenberger et al., 1998). Bennet *et al*, demonstrated that induction of OVA specific CTL response was dependent on both CD40 and CD40L

molecules and that in absence of CD4 T cells CD40 agonist Ab could substitute for the help. Schoenberger *et al*, demonstrated that CTL priming to TAA is dependent on CD40 expressed by DC and again CD40 agonist Ab could substitute for the lack of T helper cells. Similarly Ridge *et al*, demonstrated that DC stimulated in vitro with agonist Ab to CD40 can prime CTL in vitro even in the absence of CD4 T cells.

Information accumulated in the past years suggest that activated CD4 T cells trigger DC through CD40L, enhancing their capacity to produce IL-12, extending their survival and certifying their ability to activate CD8 effector T cells (Cella *et al.*, 1996; Miga *et al.*, 2001). It remains to be elucidated whether all the signals downstream to CD40 triggering are essential for CTL induction or whether one, maybe undiscovered CTL priming signal, is generated through CD40 triggering.

Lu *et al*, demonstrated the existence of CD40-independent pathways of T cell help. They hypothesized two alternative pathways, one consisting of cytokine-mediated direct cross-talk between CD4 and CD8 T cells, possibly through IL-2, and the other through a signal mediated by direct contact of CD4 T cells with the DC. The nature of this contact-mediated signal, however, was not identified (Lu *et al.*, 2000).

Helper-independent CTL responses were also found. In most cases such responses were directed against pathogens. However even if, for the generation of CD8 primary effector T cells against virus, CD4 T cell help was found to be dispensable, such “unhelped” CD8 T cells responded poorly to subsequent antigenic challenge indicating that CD4 help is necessary for a qualitative and durable CD8 memory response (Shedlock and Shen, 2003; Sun and Bevan, 2003).

In antitumor responses, CD4 T cells are important not only in CTL generation but also in maintaining the CTL pools. Indeed, a greater number of MHC class I-restricted, HA peptide reactive CD8 T cells can be recovered from animals that have been transferred with both antigen specific CD8 and CD4 T cells. The authors proposed that CD4 T cells

“help”, in maintaining the CD8 T cells activity, is particularly important when the immune system is responding to replicating target such as tumors and viruses (Marzo et al., 2000). In addition to activating T cell responses, mature DC can induce NK activation and B cell differentiation into antibody producing cells.

1.2.3 Dendritic cells and tolerance induction

Although most studies focus on the role of DC in activation of T cells, DC can also have an apparently opposite effect, the induction of tolerance [reviewed in ref. (Steinman et al., 2003)]. Before T cells encounter foreign antigens, the T cell repertoire must be shaped to delete those cells self-reactive against self-antigens. This event occurs in the thymus, and is called central tolerance

In the thymus, DC are located exclusively in the medulla and induce deletion of positively selected thymocytes. Thymic medullary epithelium also expresses high levels of MHC-class II and plays a significant role in central tolerance to self-antigens expressed by epithelial cells.

Central tolerance however is not complete. Self-reactive T cell clones with low affinity for TCR escape negative selection. Moreover, the abundant proteins present in the periphery for which tolerance is needed may not be presented in the thymus. Peripheral tolerance is consequently necessary to complement and complete thymic induced tolerance. DC have a role in the induction and maintenance of both central and peripheral tolerance (Steinman et al., 2003).

DC present self-antigens in the steady state and induce deletion or anergy of naïve T cells in the periphery. Indeed when antigens of the pancreatic islet β cells are presented in the draining LN in the steady state, clone specific CD8 T cells are deleted suggesting that the

state of DC during antigen presentation conveys immunity or tolerance (Morgan et al., 1999).

In immature DC, the secretion of cytokines such as TGF- β and IL-10 induces CD4 T regulatory cells (Tr1) that in turn suppress the immune response (Jonuleit and Schmitt, 2003). Tolerance is an obstacle to cancer immunotherapy because several TAA are inappropriately expressed self-antigens. Data from mouse studies and human clinical trials demonstrate that it is possible to elicit a CD8 T cell response to tumor antigens, suggesting that tolerance to these tumor antigens is not complete. Strategies based on recruiting DC to the tumor site and aiming at eliciting CD8 T cell response should consider the possibility that DC, biased by the tumor microenvironment, will present tumor antigens in an inappropriate costimulatory context, leading to tolerance instead of immunity (see section 1.5 for further discussion).

Figure 1.3 schematizes the discussed functions of dendritic cells in the immune system.

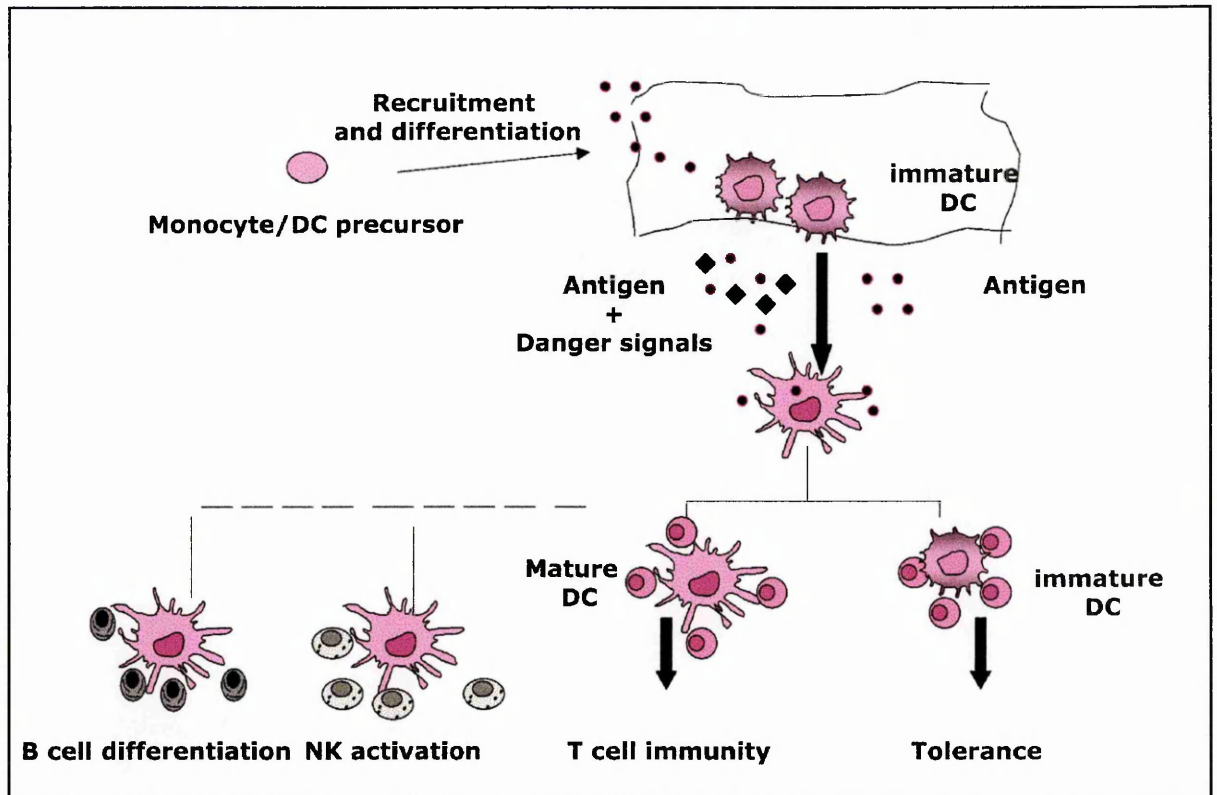


Figure 1.3 Overview of DC functions in immune responses

1.2.4 Dendritic cells-based immunotherapy

Given their central role in triggering the immune response, DC are most commonly used to stimulate T cell response against malignancies.

There is a large body of evidence showing that in animal models, DC loaded ex-vivo with tumor antigens and re-infused in the animal, lead to protection against subsequent challenges with live tumor cells and in some case rejection of pre-existing tumors (Celluzzi et al., 1996; Fields et al., 1998; Gong et al., 1997; Paglia et al., 1996; Zitvogel et al., 1996). A number of different strategies have been used to deliver tumor antigens to DC, including electroporation with naked plasmid DNA, liposome DNA complexes, peptides, tumor cell lysate and fusion with tumor cells.

These studies created the translational basis to test this approach in clinical trials. The most common approach consists in preparing large quantity of autologous DC from peripheral blood monocytes differentiated with GM-CSF and IL-4. Such DC are then loaded with tumor antigens and often matured with TNF- α before injection into patients [reviewed in ref. (Fong and Engleman, 2000; O'Neill et al., 2004; Schuler et al., 2003)].

The choice of the right tumor antigen remains the critical issue determining the success of such strategy. Tumors may resist to CD8 T cell lysis by selecting antigen-loss variants, therefore antigenic molecules necessary for the tumor growth are avidly searched.

MHC-restricted peptides modified to enhance binding to MHC-class I molecule have been used; however the most encouraging data from clinical trials come from the use of whole protein, killed tumor cells or tumor cell lysate. The advantage of these approaches is that the entire antigenic repertoire is delivered to DC for cross-presentation.

The availability of sensitive techniques to monitor the induction of T-cell responses has proved that DC-based vaccines can induce primary response to TAA. Unfortunately in the majority of these trials the presence of specific T cells response did not correlate with tumor regression [reviewed in ref. (Rosenberg et al., 2004)]. A number of reasons may explain this incongruence: 1) it is not yet clear what magnitude of T cell response correlates with protective immunity; therefore the number of the induced CD8 T cells may have been too small; 2) the induced CTL may have been ineffective in tumor killing because biased by the tumor microenvironment; 3) patients that were enrolled in many of these studies may have had a tumor load such to render the sole adaptive response insufficient for its elimination.

Strategies based on ex vivo manipulation of DC are extremely expensive and time consuming; moreover, by altering the physiology of antigen processing and presentation, such method may lose the desired efficacy. To avoid this problem, alternative approaches are available that foresee the possibility of loading DC with tumor antigen directly in vivo.

This obviates the need for DC purification and in vitro manipulation, and ensures the continuous availability of DC precursors, which upon antigen uptake, undergo migration and encounter with T cells in a more physiological manner.

An extremely efficient way to recruit DC locally is the in situ delivery of chemokines (see section 1.3 for further discussion).

DC have also been loaded directly in vivo by targeting the DEC 205 receptor expressed on their surface (Bonifaz et al., 2004) (Hawiger et al., 2001). In these studies the authors demonstrated that small amounts of DEC205-conjugated OVA antigen were capable of inducing CD4 and CD8 combined immunity, in the presence of a DC maturation stimulus (anti-CD40 triggering Ab). Interestingly, targeting the antigen via DEC 205 without maturation stimuli induced tolerance rather than immunity, strongly suggesting that effective in situ targeting strategies must be coupled with maturation stimuli to trigger immunity.

1.3 Chemokines

1.3.1 Chemokines and the trafficking of cells of the immune system

Chemokines are small molecules (9kDa) that orchestrate the circulation of leukocytes by mediating their adhesion to endothelial cells, initiation of transendothelial migration and tissue-invasion. Chemokines have also a role in angiogenesis, hematopoiesis and organogenesis [reviewed in ref. (Baggiolini, 1998; Mackay, 2001; Moser and Loetscher, 2001; Zlotnik and Yoshie, 2000)].

Members of the family have in common a repetition of four cysteins in their protein sequences. Depending on the position of the first two cysteins residues chemokines have been divided in 4 subgroups, the C-C, CXC, C and CX3C.

A high redundancy is present among the chemokines family in terms of function and receptor sharing. The chemokines that are located on the same chromosome within gene clusters have the highest redundancy in terms of function, while those mapping unclustered have mostly unique receptors and function. Such redundancy may reflect their ability to orchestrate the movement of different leukocyte subsets in complicated situations such as acute or chronic inflammation where a variety of leukocytes are abundantly present.

The biological effects of chemokines are mediated by G-protein coupled transmembrane receptors (GPCR), which are also located into clusters within chromosomes.

A recent classification has divided chemokines on the basis of their function and distinguishes them between “inflammatory” and “homeostatic” (Moser and Loetscher, 2001).

Inflammatory chemokines are produced by tissue or infiltrating leukocytes after the encounter with pathogens or in situations of inflammation. Such chemokines recruit monocytes, granulocytes and effector T cells at the site where they are released.

On the contrary, homeostatic chemokines are endowed of more “housekeeping” functions such as trafficking and homing of lymphocytes, assuring their homeostatic recirculation (Moser and Loetscher, 2001).

Monocytes and DC when immature use receptors for inflammatory chemokines such as CCR1, 2, 5, 6 and 9 to enter peripheral tissue.

Upon entering in peripheral tissues and exposure to maturation stimuli, such as pathogens or other stimuli delivered by innate effectors cells, DC down regulate inflammatory receptors and upregulate the “lymphoid” receptor CCR7 that allows DC to respond to CCL19 and CCL21 (Mackay, 2001; Zlotnik and Yoshie, 2000).

CCR7 is important in the circulation of T cells and its expression discriminates between homing to the secondary lymphoid organs and peripheral tissues.

Naïve T cells express CCR7 that assure the retention in lymphoid organs where the immune response is mounted. Once activated, antigen specific T cells lose CCR7 expression and use other chemokines to reach the site of inflammation. Indeed, CCR7 deficient mice not only show strong defect in DC homing to LN but have significantly reduced number of naïve T cells in lymphoid organs. As a consequence of both DC and T cells homing defects, CCR7-deficient mice have impaired immune responses such as delayed-type hypersensitivity reaction (DTH) and antibodies production (Forster et al., 1999; Randolph et al., 1999). A number of new evidences have demonstrated that chemokines not only orchestrate leukocytes circulation but act on the target cells to modify their effector functions. For example, the human CC chemokine CCL5 is able to co-stimulate cell proliferation in response to anti-CD3 triggering. Such effect depends on the presence of IL-2. Indeed IL-2 up regulates CCR5 expression on T lymphocytes, providing an additive effect. Interestingly other human CC chemokines that use CCR5 receptor have similar activity (Taub et al., 1996). SDF-1 α can also co-stimulate human CD4 positive T cells in response to anti-CD3 triggering, by augmenting production of IL-2, IL-4 and IFN- γ (Nanki and Lipsky, 2000; Suzuki et al., 2001).

1.3.2 Chemokines in cancer immunotherapy

Engineering tumor cells to express immunostimulatory molecules is one of the most popular approaches to study the role of such molecules in tumor rejection and to generate cell-based vaccines.

A large number of studies (the most important ones are summarized in Table 1.1) use chemokine genes, given the ability of the corresponding proteins to recruit leukocytes and to enhance certain functions [reviewed in ref. (Homey et al., 2002)].

Beside their role in anticancer strategies this method also addresses the biological function of chemokines *in vivo*.

The different approaches have been used: transduction of tumor cells with chemokines genes *in vitro* and their transplantation *in vivo*, delivery of chemokines directly *in vivo* in pre-established tumors and combination of chemokines with other immunomodulatory molecules. The following paragraphs will summarize such studies.

In many cases chemokine gene transduction reduced or completely abrogated tumorigenicity of tumor cell lines by a T cell dependent mechanism, as demonstrated by experiments performed in T cells depleted mice (Homey et al., 2002).

Antitumor response chemokine-induced is not only mediated by T cells, but cells of the innate response are often involved and angiostatic effects are important as well. For example antitumor mechanisms mediated by hCCL16 were found to be dependent on the presence of polymorphonucleated cells (PMN) within the tumor (Giovarelli et al., 2000).

Neutrophil accumulation mediated tumor destruction of SP0/2 myeloma cell transfected with XCL1 although complete regression required a T cell response, indicating that this chemokine exert antitumor activity by both innate and effector mechanisms (Cairns et al., 2001).

CXCL10 and CXCL9 share the same receptor (CXCR3) and are chemotactic *in vitro* for T lymphocytes and NK cells (Loetscher et al., 1996). Both chemokines have a strong inhibitory effect on angiogenesis (Angiolillo et al., 1995). Indeed, CXCL10- and CXCL9-gene transduction into tumor cell lines resulted in tumor rejection, an effect thought to result from their action on lymphocytes as well as their anti-angiogenic properties (Arenberg et al., 1996) (Sgadari et al., 1996) (Sgadari et al., 1997).

Results obtained with this approach are not always consistent. For instance CCL2-gene transfer resulted in macrophage recruitment and, as a consequence, had no effects on (Hirose et al., 1995), tumor destruction, (Huang et al., 1994b), partial inhibition of tumor

(Laning et al., 1994) (Bottazzi et al., 1992) or augmentation of tumor growth and of metastatic spreading (Bottazzi et al., 1992) (Nesbit et al., 2001) (Nakashima et al., 1995). Nesbit *et al*, have elegantly investigated these contradictory results by transducing tumor cell line with an adenoviral vector at different MOI such to obtain different level of CCL2 expression. They demonstrated that the effect of CCL2 on tumor growth is dose dependent and related to the degree of leukocyte infiltration. Indeed, low level of CCL2 increased tumor growth, while higher-level provoked tumor destruction throughout massive macrophages infiltration. Macrophages recruited by low CCL2 promoted tumor growth because of macrophages effect on angiogenesis and as supporter of tumor stroma. Interestingly, tumors producing low CCL2 level had the macrophages located at the edge of the tumor area suggesting that their peritumoral localization is beneficial for the tumor (Nesbit et al., 2001).

As mentioned in section 1.2, strategies that aim at enriching DC at the tumor site have shown some promising results. This approach is based on the concept that DC chemoattraction directly at the tumor site can favour uptake of tumor derived antigens.

In this context interesting results came from the use of CCL20 and CCL21, both effective in attracting DC in vitro. Vicari *et al*, showed that mouse CCL21 delayed tumorigenicity of transfected C26 colon carcinoma cell through angiostatic and CD8 T cell mediated mechanisms (Vicari et al., 2000). Although tumors were infiltrated by a large number of granulocytes, their depletion did not abrogate tumor rejection, while NK cells were found necessary. Because of the ability of CCL21 to recruit both naïve T cells and DC, the authors suggested that T cell priming could took place within the CCL21-expressing tumor rather than in its DLN. This hypothesis is supported by the notion that CCL21 can promote the development of an ectopic lymphoid tissue (Fan et al., 2000). Formal proof of this hypothesis came from a more recent study from Kirk *et al* (Kirk et al., 2001a). These authors used BM derived DC infected with an adenoviral vector expressing CCL21 and

injected them intratumorally in the attempt to mount a specific T cell response. Interestingly such modified DC were unable to reach the DLN, but promoted an accumulation of CD4 and CD8 T cells at the tumor site. By using mice lacking secondary lymphoid organs ($Lt\alpha^{-/-}$), the authors demonstrated that the priming of T cells by DC took place inside the tumor that became the site of T cell triggering and, as well, T cells target. Indeed, in $Lt\alpha^{-/-}$ mice, activated T cells were recruited at the tumor site and antitumor activity, manifested as delayed tumor growth, was comparable to that of wt mice (Kirk et al., 2001a).

CCL21 was found to be effective as an antitumor agent when injected into prestablished tumors as recombinant protein. In two studies multiple intratumoral injection of recombinant protein induced 40% complete tumor regression (Sharma et al., 2000) and significantly delayed tumor growth (Kirk et al., 2001b).

In the same vein, Fushimi and co-authors injected adenovirus-encoding CCL20 into prestablished tumors and reported a cure rate between 70 and 50%, depending on the tumor model used. Inflammation and enlargement of DLN, was accompanied by accumulation of DC; however authors did not investigated whether such DC-infiltration was the consequences of a general inflammation or whether they migrated from the tumor. Tumor rejection was reported to involve exclusively T cell response, but the presence/role of innate immunity was not investigated (Fushimi et al., 2000).

Chemokines seem to have limited therapeutic potential when used as single agent against well-established tumors. Therefore, many approaches have combined the chemoattractant proprieties of chemokines with other immunomodulatory molecules such as cytokines, or TLR ligand CpG (Homey et al., 2002).

For example an adenoviral vector expressing XCL1 was ineffective when used alone against pre-established mammary carcinomas, but when co-expressed with IL-2 or IL-12, induced tumor regression in 40 to 60% of treated mice (Emtage et al., 1999).

In a separate study, an adenoviral vector delivering XCL1 was combined with adoptive T-cell therapy (Huang et al., 2002a) to treat pre-established SP2/0 myeloma. The authors prepared SP2/0 tumor-specific T lymphocytes by isolating T cells from mice that rejected SP2/0 cells transfected in vitro with XCL1. T cells were then expanded in vitro and re-injected in vivo in combination with intratumoral administration of the adenovector expressing XCL1. Neither adenovirus-mediated XCL1 gene transfer alone nor adoptive T-cell transfers cured tumor-bearing mice. In contrast, by combining the two treatments, six of eight mice were cured indicating the synergistic therapeutic effects of locally expressed XCL1 with adoptive T-cell therapy. The study shows that tumor-specific activated T lymphocytes expressed XCR-1 mRNA and migrated in response to XCL1 in vitro. Moreover the authors demonstrated an enhanced tumor infiltration of adoptively transferred tumor-specific T cells in XCL1 treated tumors, supporting the hypothesis that the observed antitumor activity is derived from the combined chemotactic and stimulatory effects of XCL1 on adoptively transferred activated T cells (Cairns et al., 2001).

Narvaiza *et al*, combined an adenovirus expressing CXCL10 with one expressing IL-12 a strategy that allowed lowering the dose of effective IL-12, preventing its toxicity, while improving the therapeutic efficacy of the T cell response. The antitumor response was observed only when the two agents were delivered together in the same tumor nodules, supporting the so called “attraction and activation hypothesis” which predicts the need of activating the chemokine-recruited leukocytes to obtain an optimal antitumor response (Narvaiza et al., 2000).

Table 1.1

CHEMOKINE	MODEL	INFILTRATING LEUKOCYTES	IMMUNE RESPONSE /OUTCOME	REF
m/h CCL2	Gene transfer into CHO cell line. Comparison with other h/m CCL	Neutrophils	No tumor rejection or reduce growth	(Hiros e et al., 1995)
hCCL2	Gene transfer into B16 melanoma model.	Macrophages	Slower growth rate when cells are injected s.c.	(Botta zzi et al., 1992)
mCCL2	Gene transfer into CT26 colon carcinoma model.	N.D.	Reduce number of metastases following i.v. injection of modified cells.	(Huan g et al., 1994b)
hCCL2	Gene transfer into C26 colon carcinoma.	N.D.	Increase number of metastases upon i.v. injection. The authors conclude that CCL2 augment angiogenesis	(Nakas hima et al., 1995)
hCCL2	Gene transfer into SBcl2 melanoma cell line via adenoviral vector to obtain different amount of production of CCL2.	Macrophages	Low level of CCL2 at the time of tumor take led to increase tumorigenesis whereas an higher production to a massive infiltration of macrophages and tumor destruction.	(Nesbi t et al., 2001)
mCCL1	Gene transfer into IgG and IgA myeloma.	Macrophages and neutrophils	Reduce tumorigenesis local necrosis	(Lanin g et al., 1994)
hCXCL8 mCCL3 hCCL3	Gene transfer into C26 colon carcinoma.	Macrophages and neutrophils	Reduced tumorigenesis of cells transfected with h/m CCL3 but not with hCXCL8.	(Nakas hima et al., 1996)
hCCL5	Gene transfer into murine fibrosarcoma.	Macrophages	Complete rejection. Complete abrogation of rejection in mice depleted for CD8 T cells. Only partial need of CD4 T cells.	(Mule et al., 1996)

Table 1.1

CHEMOKINE	MODEL	INFILTRATING LEUKOCYTES	IMMUNE RESPONSE /OUTCOME	REF
hCCL16	Gene transfer into murine TSA adenocarcinoma.	DC, CD8, CD4 and macrophages	Complete rejection dependent on CD8 T cells and PMN.	(Giovarelli et al., 2000)
hCCL19	Retrovirus infection of C3L5 breast carcinoma cell line.	N.D.	Tumor rejection of CCL19 transfected cells. NK and CD8 mediated. Same cells used as vaccine showed low therapeutic activity.	(Braun et al., 2000)
XCL1	Gene transfer into SP2/0 myeloma model.	Mainly Neutrophils	T cells are needed for total rejection but in their absence the only presence of neutrophils was sufficient to significantly delay tumor growth	(Cairns et al., 2001)
XCL1	Injection of adenovirus expressing XCL1 in preestablished SP/20 myeloma tumor combined or not with adoptive T cell transfer.	Activated T cells	Strong synergy of XCL1 expression at tumor site with adoptive T cell therapy. XCL1 attract effector T cells at tumor site.	(Huang et al., 2002a)
MCP-3	Gene transfer into P815 mastocytoma cell line.	Macrophages, neutrophils accumulate within the tumor while DC peritumorally	Tumor rejection of modified cells. T cell depletion and IFN- γ blocking abrogated rejection.	(Fioretti et al., 1998)
CCL3	Gene transfer into B16 melanoma model.	N.D.	CCL3 expression had no effect on s.c. injection of modified tumor cells while significantly inhibited the ability of modified cells to form metastases in a CD8 dependent fashion.	(van Deventer et al., 2002)

Table 1.1

CHEMOKINE	MODEL	INFILTRATING LEUKOCYTES	IMMUNE RESPONSE /OUTCOME	REF
CCL5	Gene transfer into EL4 tymoma cell line and intratumoral injection of plasmid encoding CCL5-Ig fusion protein for therapy	T cells and NK	Decreased tumorigenicity of modified cell lines and decreased tumor growth when used in a therapeutic setting. T cell mediated.	(Lavergne et al., 2004)
CCL21	Gene transfer into C26 colon carcinoma model.	DC, monocytes, granulocyte	Reduced tumorigenesis by angiostatic and CD8 mediated T cell response.	(Vicari et al., 2000)
CCL21	Multiple i.t. injection of recombinant CCL21 in 3LL and LIC2 lung carcinoma.	DC, CD4 and CD8 T	Reduced tumor growth and 40% of complete regression.	(Sharma et al., 2000)
CCL21	Multiple i.t. injection of recombinant CCL21 in B16 tumor plus vaccine consisting of DC expressing SLC.	DC, CD4 and CD8 T	Decreased tumor growth after direct injection of rCCL21	{Kirk, 2001 #26
CCL21	DC genetically modified via adenoviral vector inoculated into preestablished B16 melanoma tumor model.	CD4 and CD8	Inhibition of growth of preestablished tumors. Rapid influx of T cells secreting IFN- γ into treated tumors. Priming of T cells is independent from DLN.	(Kirk et al., 2001a)
XCL10	Combination of adenovirus expressing XCL10 with one expressing IL-12.	Lymphocytes infiltration	Strong synergy observed only when the two molecules were delivered at the same tumor site. CD4 and CD8 were needed.	(Narvaiza et al., 2000)

Table 1.1

CHEMOKINE	MODEL	INFILTRATING LEUKOCYTES	IMMUNE RESPONSE /OUTCOME	REF
CCL20	Intratumoral injection of adenovirus expressing CCL20. Three tumor model used: CT26 (colon carcinoma), B16 (melanoma) and LLC (lung carcinoma)	DC infiltrates treated tumor and their DLN	40-60% of rejection rate. T cells are needed.	(Fushimi et al., 2000)

Table 1.1 Some models of chemokine treated tumors. Abbreviation used i.t., intratumoral; PMN, polymorphonuclear leukocyte; ND, not determined; h/m human and mouse.

1.3.3 The biology of CCL16

Liver-expressed chemokine CCL16, also known as LEC, novel chemokine 4, human CC chemokine 4, human CC chemokine 1, was originally found as expressed sequence in a tag library, and its gene was then mapped in the CC chemokine cluster on chromosome 17 (Fukuda et al., 1999; Naruse et al., 1996).

CCL16 was found to be chemotactic for human lymphocytes, monocytes and DC. It was reported to bind to CCR1 with the highest affinity but also to CCR2, CCR5 (Nomiyama et al., 2001) and CCR8 (Howard et al., 2000).

Two CCL16 transcripts of 1,5 kb and 0.5 kb have been described to be expressed at the same level in the normal human liver (Shoudai et al., 1998). Both messages encode a protein of 120 aa. Few reports have investigated its biological role.

Hedrick *et al.*, demonstrated that the CCL16 shorter message is expressed at weak level by human lymphocytes, including NK cells and $\gamma\delta$ T cells. The highest level was detected in human monocytes activated by LPS and IFN- γ . Interestingly when monocytes were activated by these agents in the presence of IL-10, the longest message was found to be upregulated. The biological significance of this upregulation remains unclear but the authors proposed that CCL16 act as an anti-inflammatory chemokine (Hedrick et al., 1998). However, recent data suggest that it acts as inflammatory chemokine. Indeed, CCL16 enhances the ability of murine macrophages to phagocyte and their antigen presenting cell function. Small amount of recombinant protein (10-100 ng) triggers the killing activity of macrophages against a mammary carcinoma cell line, TSA, via increased expression of TNF- α and FasL. Moreover CCL16 induced macrophages to release downstream mediators such as CCL2 and CCL5 and inflammatory cytokines such as TNF- α , IL-1 β and IL-12 (Cappello et al., 2004).

It has been proposed that IL-10 secretion can increase inflammation as a results of upregulated CCL16 expression that in turn leads to a massive enhancement of CCL2 release (Cappello et al., 2004). CCL2 is an inflammatory chemokine that is upregulated in various types of hepatic injury, and in this environment IL-10 synergizes with CCL16 to upregulate its expression. A recent study analyzes CCL16 expression in human biopsy of Ulcerative Colitis (UC), an inflammatory disease associated with high level of IL-10 expression. Abundant inflammatory infiltrate can be detected within the lesions where macrophages were found to express high level of CCL16. The immunohistochemical and ultra structural characteristics of the lamina propria in UC suggest that IL-10 mediated up regulation of CCL16 is responsible for the inflammatory reaction associated with these lesions (Pannellini et al., 2004). Therefore it seems likely that the peculiar pro-inflammatory activity of IL-10 in certain pathological conditions can be explained by the upregulation of CCL16 that leads to leukocyte recruitment and induction of a further inflammatory cytokines and chemokines cascade.

Finally, in human, CCL16 was found to promote angiogenesis in 3 different ways 1) by inducing endothelial cell motility, 2) by inducing the release of proinflammatory and proangiogenic chemokines, such as CCL2 and CXCL18 and 3) by sensitizing endothelial cells to otherwise ineffective concentration of VEGF (Strasly et al., 2004).

1.4 TLR9 triggering via CpG ODN in cancer immunotherapy

1.4.1 Mechanisms of action

The innate immune system is activated by exposure to pathogen-associated molecular patterns (PAMP) expressed by infectious microorganism.

The triggering of the innate response contrasts with the early proliferation of the infectious agent and stimulates the induction of the adaptive response that not only clears the pathogens but retains the memory of their encounter. The recognition of PAMP is mediated by the already mentioned TLR.

Unmethylated DNA in a particular sequence context (CpG motifs) constitutes one of the most potent PAMP. Because bacteria cannot methylate cytosine residues and most of the mammalian genome is methylated, CpG motifs in bacterial DNA are recognized as diverse, such to alert the immune system about the pathogen presence.

Cells of the innate response recognize the CpG motifs using TLR9 receptor. Upon binding to CpG the TLR9 TIR domain recruits the adapter protein MyD88 that in turn recruits the IL-1 receptor-associated kinase (IRAK) and TNF receptor-associated factor 6 (TRAF6) to the TLR9 complex, leading to the activation of transcription factors such as NF- κ B, AP1, CEBP and CREB. These factors activate the transcription of a number of inflammatory cytokines and chemokines genes that are the final mediators of CpG effect on the innate system. To the activation of the innate system follows a strong activation of the adaptive response; indeed CpG motifs, among the various PAMP, appears to induce the strongest Th-1 type immune response [reviewed in ref. (Klinman, 2004; Verthelyi and Zeuner, 2003)].

Mice and humans differ for the type of cells, that expressing TLR9, are able to respond to CpG motifs. In mice, TLR9 is expressed by murine plasmacytoid and non-plasmacytoid DC regardless their spleen, thymus or bone marrow origin. In mice also

monocytes/macrophages respond to CpG. On the contrary, in human CpG response is restricted to plasmacytoid DC (pDC) and B cells.

In mice, optimal immune activation requires a CpG motif in which an unmethylated CpG dinucleotide is flanked by two 5' purines and two 3' pyrimidines. The cascade of events activated by TLR9 leads to increase antigen presenting functions of DC and secretion of inflammatory chemokines/cytokines by DC, macrophages and NK cells (Verthelyi and Zeuner, 2003).

Interestingly, among a number of different TLR ligands, CpG ODN seems the only able of increasing the ability of murine BM derived DC to cross-present exogenous antigens, demonstrating a peculiarity of CpG in affecting the antigen-processing machinery (Datta et al., 2003).

In humans, two types of immunostimulatory sequences, the K and the D, have been described to have different effects. The major differences among the two are on pDC. K ODN act on pDC to promote TNF- α synthesis, to increase survival and up regulation of maturation markers (CD40, CD80, CD86) but induces low level of IFN α/β . On the contrary, the D type promotes large amount of IFN α/β secretion that in turn promotes secondary effects on other immune cell types (Verthelyi and Zeuner, 2003). For example pDC release of type I IFN promotes secretion of CXCL10 by human monocytes (Blackwell and Krieg, 2003), mediates monocytes differentiation into DC (Hartmann et al., 1999) and induces monocytes to secrete TNF-related-inducing ligand (TRAIL) enabling them to kill tumor cells (Kemp et al., 2003). Recently a new class of CpG ODN (class C) was shown to elicit both human B cells and pDC activation associated with release of high amount of types I IFN (Marshall et al., 2003).

1.4.2 Antitumor applications of CpG oligodeoxynucleotide.

The described ability of CpG to activate the immune system has found important therapeutic applications in the fields of vaccine development, protection by infectious organisms and treatment of cancer.

Figure 1.3 schematizes the field in which synthetic CpG have been shown to have potential therapeutic effects.

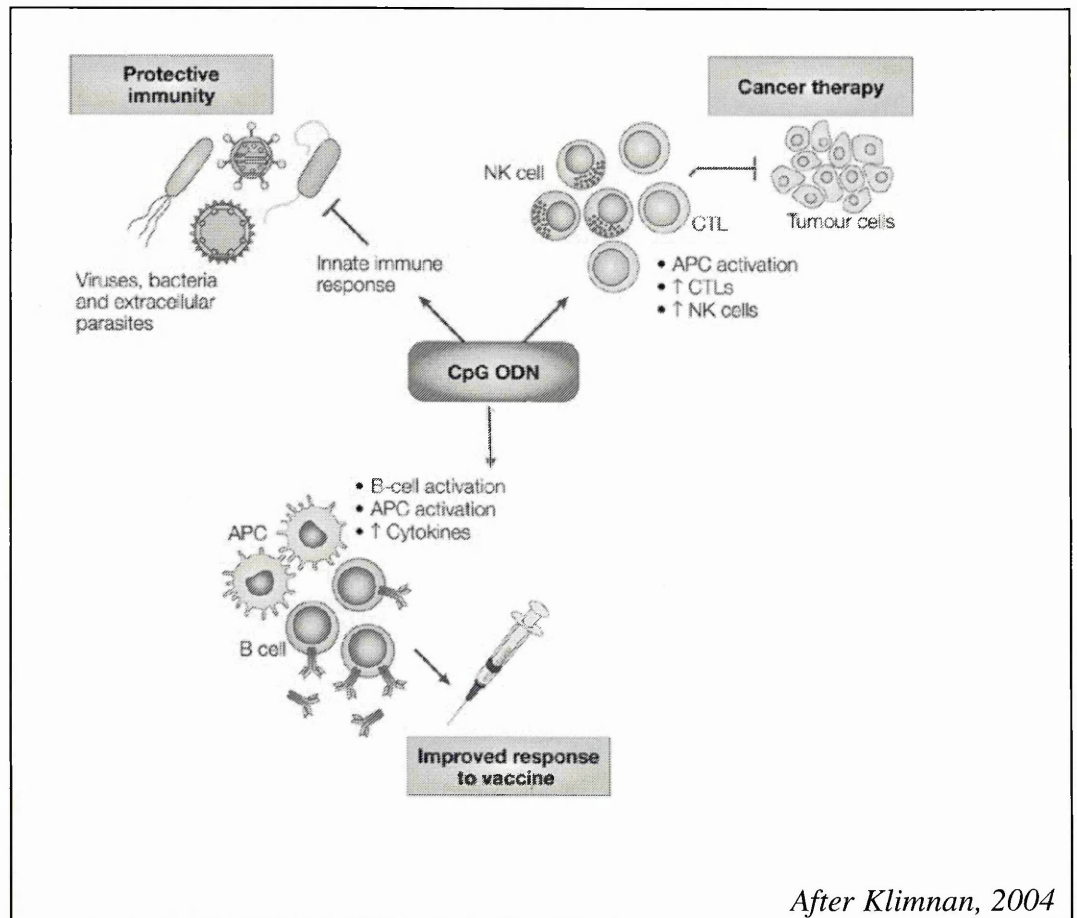


Figure 1.4 CpG alone have been shown to induce protective immunity against pathogens.

Because of its effect on antigen presenting cell functions and B cell activation, CpG ODN creates a cytokine/chemokine milieu that enhances the effectiveness of co-administered vaccines. The cascade of events associated with intratumoral administration of CpG has been shown to induce tumor rejection to a certain degree.

Several applications of CpG in cancer immunotherapy have been described either in settings of prevention, as adjuvant of tumor vaccines or of therapy to treat already established tumors [reviewed in ref. (Klinman, 2004)].

In prevention settings, when incorporated into DNA or peptide based antigen-specific vaccines, CpG were shown to protect against subsequent tumor challenge (Huang et al., 1997) (Miconnet et al., 2002; Stern et al., 2002).

The efficacy of CpG monotherapy to cure pre-established tumors appears to vary dramatically depending on the tumor model used and the number of administrations (Krieg, 2004) (see table 1.2).

In a highly immunogenic tumor model of cervical cancer, complete regression was observed if repeated systemic administrations were started two days after injection, while lower (50%) regression was obtained when treatment began ten days after tumor inoculation. Interestingly, CD8 but not CD4 T cells were needed for the observed therapeutic effect. In this tumor model CD4 appeared to function as immunosuppressive cells, as CD4 KO mice showed increase antitumor effect of CpG in comparison with wt mice (Baines and Celis, 2003).

Systemic administration of CpG was shown to delay carcinogenesis in a model of spontaneous mammary carcinogenesis (FVB-NeuN). Transgenic female were treated weekly from the 10th week of age with CpG. Tumor incidence and number of tumors/mouse were significantly lower in treated mice compared with the control group, indicating that by only maintaining the immune system on alert, carcinogenesis can be impaired. However, no complete regression or memory response against tumor challenges was obtained, consistent with the notion of a predominant or exclusive role for the innate immunity in the observed inhibitory effect.

Peritumoral or intratumoral administration of CpG was found to be more effective than its systemic administration (Heikenwalder et al., 2004; Kwarada et al., 2001). Sfondrini *et al*,

studied the effectiveness of CpG given locally in relation to the immunogenicity of the treated tumor. Repeated intratumoral injections of CpG into B16 tumor leads to reduced tumor growth and expansion of IFN- γ -producing specific CD8 T cells. However NK rather than CD8 T cells were found to be responsible for the antitumor activity. The authors suggested that because B16 melanoma is a highly aggressive tumor and expresses low level of MHC class I molecule, the CTL response mounted after CpG treatment is insufficient to block tumor growth and to completely reject growing tumors (Sfondrini et al., 2002).

Of great interest are studies that combined TLR9 stimulation with other therapeutic agents such as other immunostimulatory molecules, adoptive T cell transfer or chemotherapy.

Furomoto *et al*, found that multiple intratumoral administrations of CpG into B16 tumor are not able to halt tumor growth while its combination with rCCL21 induces a potent systemic antitumor response. The authors demonstrate that both the number of DC infiltrating the tumor and their activation state were important for an optimal induction of T cell response especially when poorly immunogenic tumors were treated. Indeed, CCL20 alone was able to reject CT26 tumor that contrary to B16 tumor has functional infiltrating DC that therefore do not need further stimuli like CpG to become activated (Furumoto et al., 2004).

In another study TLR9 triggering was combined with Ag-specific immunization to cure spontaneous tumors in RIP-Tag transgenic mice. Vaccination with antigen-encoding plasmid DNA elicited specific CTL that however were unable to infiltrate the tumors. The number of elicited CTL was not the limiting factor since transfer of high number of pre-activated specific CD8 T cells gave similar results. Combination of pre-activated CD8 and CD4 T cells with CpG proved to be highly effective, because CpG rendered the tumor permissive for massive leukocytes infiltration and destruction (Garbi et al., 2004).

Weigel *et al*, demonstrated that CpG or chemotherapeutic agents, cyclophosphamide or topotecan, given as single treatments, could not induce rejection of well-established rhabdomyosarcoma tumors. On the contrary when chemotherapy was combined with systemic or local administration of CpG, 15 to 40% of mice rejected the tumor, suggesting that chemotherapy releases tumor antigens that are better presented by TLR9 stimulated DC. Antitumor activity required T cells but not NK cells suggesting a role for T cells in controlling the residual tumor remnants left by the chemotherapy (Weigel et al., 2003).

These studies, together with many others in the literature, strongly suggest that the outcome of TLR9 stimulation varies depending on the type of tumor, its size, the level of MHC molecules expression, the susceptibility to the immune effector cells as well as the status of responsiveness of the infiltrates to the triggering, and clearly indicate that TLR9 may strongly synergize with other immunomodulatory therapies towards tumor rejection.

(Table 1.2 represents a summary of murine tumor models treated with CpG ODN).

Table 1.2

Tumor Model	Schedule of treatment	Outcome	Ref
C3 cervical cancer model	Systemic injection of CpG starting day 2 or day 10 post tumor challenge.	Complete tumor rejection if treatment started at day 2 and 50% if started at day 10. CD8 T cells are needed but not CD4.	(Baines and Celis, 2003)
FVB NeuN transgenic mice	Weekly injection of 20 μ g of CpG starting at the 10 th week of age.	Delayed tumor onset. Accumulation of macrophages infiltrate at the site of mammary gland. No complete rejection or memory response was elicited.	(Sfondrini et al., 2002)
AG104A, IE7 fibrosarcoma, B16 melanoma and 3LL lung carcinoma models	Compare the effectiveness of systemic versus peritumoral administrations of CpG. Schedule: 8 injection of 20 μ g given twice a week for four weeks.	Systemic administration had only minor effects whereas intratumoral injection led to complete rejection. Introduction of the immunogenic antigen, OVA, ameliorated rejection rate and induced a stronger memory response. CD8 and NK cells were needed.	(Kawarada et al., 2001)
C26 colon and RENCA carcinoma models	Correlation between injection site and effectiveness of CpG treatment. CpG were given intratumoral or on opposite flanks starting 5 days after tumor inoculation (100 μ g weekly for 4 weeks).	CpG given alone or with irradiate tumor cells on the opposite flank of tumor challenge reduced tumor growth while intratumoral administration led to complete tumor rejection in 70% of treated mice. Long-term memory. Major contribution of CD8 T cells. Innate effector were needed although have not been investigated in details	(Heckelsmiller et al., 2002)
RM lymphoma model	3 injection of 20 μ g on day 5, 8, 11.	Tumor rejection. CD8 and NK mediated.	(Lonsdorf et al., 2003)

Table 1.2

Tumor Model	Schedule of treatment	Outcome	Ref
B16 melanoma	5 daily injection starting 5 days after tumor inoculation.	Reduced tumor growth with 10-20% of complete rejection. No memory response detected. NK rather than CD8 T cells were involved in the antitumor activity.	(Sfondrini et al., 2004)
B16 and CT26	rCCL20 (100ng daily injection for three weeks) was combined with 5 injection of CpG (80 µg each) starting 12 days after tumor inoculation.	In B16 tumor model combination treatment led to 50 % tumor rejection. CpG activate TIDC recruited by CCL20. CD8 T cells but not NK or CD4 are needed for antitumor activity.	(Furumoto et al., 2004)
RIP1-Tag5 transgenic mice spontaneous model of pancreatic carcinoma	50 µg of purified Tag protein in CFA starting at 6 weeks of ages and repeated every two weeks. 50 µg CpG-ODN combined with 2.5 x 10 ⁶ Tag specific activated CD8 or CD4 T.	Between 50-60% long-term survival when CpG was given together with Tag antigen in a preventive setting. Uptake of CpG by tumor resident macrophages changes the tissue microenvironment to support effectors cell extravasion.	(Garbi et al., 2004)
76-9 Rhabdomyosarcoma	100 µg of CpG ODN were given on day 9, 12, 16, 19. 22. 26 after tumor injection either systemically or intratumorally, chemotherapeutic agent cyclophosphamide or topotecan (200mg/kg) on day 9 and 19.	CpG or chemotherapy alone were not able to induce tumor rejection while combination resulted to 50% tumor rejection. T cell dependent mechanism.	(Weigel et al., 2003)

Table 1.2. Some models of CpG-treated tumors. Abbreviation used: CpG ODN (CpG oligonucleotide), TIDC (tumor associated dendritic cells)

1.5 Tumor escape mechanisms

1.5.1 The cancer immunosurveillance hypothesis

Paul Ehrlich, in 1909, was one of the first to formulate the hypothesis that the immune system can repress tumors that otherwise would occur at a greater frequency.

Fifty years later the demonstration that mice could be immunized against syngenic implants of tumors induced by carcinogens, virus or spontaneously arisen, proved the existence of tumor associated antigens and provided the foundation for the “cancer immunosurveillance” hypothesis formulated by Macfarlane Burnet and Lewis Thomas in 1957. Burnet defined the immunosurveillance concept as follows: *“In large, long lived animals, like most of the warm-blooded vertebrates, inheritable genetics changes must be common in somatic cells and a proportion of these changes will represent a step toward malignancy. It is an evolutionary necessity that there should be some mechanism for eliminating or inactivating such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character”* (Burnet, 1970).

The fundamental of such hypothesis is that lymphocytes act as sentinels in recognising and eliminating developing transformed cells that arise to a frequency similar to infection with pathogens [reviewed in ref. (Dunn et al., 2002; Pardoll, 2003)].

A prediction of this hypothesis is that immunodeficient individuals would develop cancer at a much higher frequency. However, epidemiological studies performed in 1970s-1980s on individuals affected by immunodeficiencies, found that although cancers such as lymphoma (Epstein-Barr origin) and Kaposi sarcoma (Herpesvirus 8 origin), developed in these individuals to a much higher frequencies, epithelial cancers such as colon, lung and prostate cancer, were not increased. This suggested that only cancers induced by viral infection are controlled by the immune system. However it should be taken into account that the majority of immunosuppressed patients tend to die in their forties raising the

possibility that not-pathogens associated cancers may have not been detected (Pardoll, 2003). Indeed, a number of recent experimental studies performed in animals suggest that the immune system is able to control tumor outgrowth. One of the most informative studies was performed using RAG2^{-/-} mice, which lack T, B and NKT cells. As a consequences of subcutaneous injections of the chemical carcinogen 3'methylcholantrene (MCA), RAG deficient mice developed sarcomas at the injection site with greater frequency than wt mice, suggesting that cells of the immune system controlled tumor development. Moreover, tumors developed in immunosufficient mice were less immunogenic and more aggressive then those in immunodeficient hosts, suggesting that the immune system favours the outgrowth of tumors that are more capable of escaping its control (Shankaran et al., 2001). This implies that the immune system not only plays a protective role against cancer in the first stages of transformation, but at later stages takes active part in its progression. Therefore the "cancer immunosurveillance" hypothesis was reformulated by the same authors under the name of "cancer immunoediting" hypothesis (Dunn et al., 2002).

Cancer immunoediting consist of three phases. 1) elimination, that is the phase in which cells of the immunosurveillance network, innate and adaptive, destroy the tumor; 2) the equilibrium phase in which tumor cells are in a state of chronic maintenance and in which tumor variants are selected to survive the immune attack; 3) the escape phase in which tumors evade the immune system control and grow in an uncontrolled manner (Fig. 5.1).

A number of tumor escape mechanisms have been described including down regulation of tumor-associated antigens, induction of immune un-responsiveness, via anergy or clonal deletion of effector T cells, expansion of CD5⁺CD25⁺ regulatory T cells and paralysis of the tumor infiltrating leukocytes.

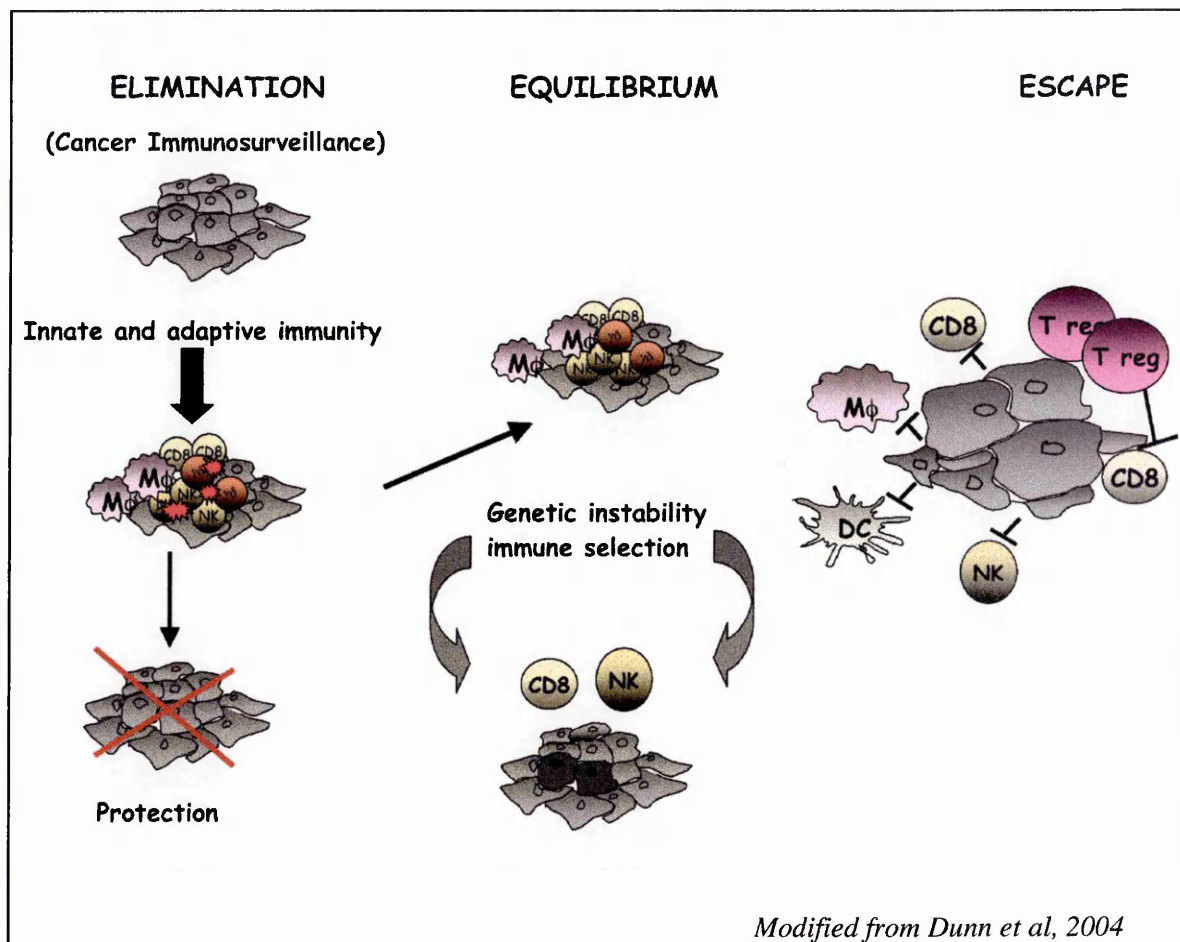


Figure 1.5 Schematic representation of three phases of the cancer immunoediting process.

The next sections are focused on the mechanisms by which infiltrating leukocytes, in particular macrophages and DC, are biased by the tumor to favour its own growth.

1.5.2 Alternative activated tumor-associated macrophages

The immunological function of macrophages has been well documented. They are released by the bone marrow as immature monocytes and circulate in the undifferentiated state before extravasion into tissue, where they differentiate into macrophages. Macrophages are abundantly present at the site of inflammation and infections, where they orchestrate the recruitment of other immune cells, contribute to destroy pathogens, and promote tissue repair. Macrophages represent a major part of the leukocytes infiltrating human tumors and in some cases up to a 1:1 ratio between tumor cells and infiltrating macrophages was observed. These cells are often referred as tumor infiltrating macrophages (TAM). TAM are thought to originate from blood monocytes recruited in the tumor, rather than from resident macrophages present in the tissue before tumor development.

TAM infiltrate a number of different human tumors such as breast, prostate, ovarian and cervical cancer, where their number correlates with poor prognosis. In only 10% of the analyzed biopsies the presence of TAM correlate with tumor destruction and good prognosis.

A number of factors produced directly by the tumor or by infiltrating leukocytes and stromal cells, such as chemokines CCL2, CCL8, CCL3 and colony CSF-1 recruit circulating monocytes and stimulate their differentiation into macrophages [reviewed in ref. (Murdoch et al., 2004; Pollard, 2004)]. Of note, in humans, tumor expression of CSF-1, which promotes differentiation from monocytes to macrophage and their proliferation, correlates with poor prognosis. Therefore tumors are able to co-opt some of the normal functions of macrophages promoting their own growth and spread of metastases.

Experiments performed in murine models of spontaneous carcinogenesis confirm this interpretation. Lin *et al*, used mice that develop spontaneous mammary tumors due to the expression in the mammary gland of the polyoma-middle T oncoprotein (PyMT mice) and crossed them with CSF-1 null mice. The resultant mice had strong defects in macrophages accumulation at the tumor site. While the initial tumor growth was similar to that of CFS-1 sufficient mice, tumor progression and metastatic ability was greatly reduced, suggesting a role of infiltrating macrophages in supporting tumor progression rather than its early formation (Lin *et al.*, 2001).

Mantovani and collaborators divided macrophages into two major subclass, named M1 and M2, and referring to the two opposite functional activities of macrophages [reviewed in *ref.* (Mantovani *et al.*, 2002)]. Different patterns of cytokines expression identify the two classes of macrophages. M1 macrophages express IL-12, TNF- α , IFN- γ and have potent tumoricidal activity while M2, also called alternative activated macrophages, typically express IL-10 and IL-1 α antagonist (Mantovani *et al.*, 2002) (Mantovani *et al.*, 2004).

The arginine metabolism in M1 macrophages is characterized by high level of inducible nitric oxide syntase (iNOS) that promotes its conversion to molecules with cytostatic activities, while in M2 macrophages generates polyamines that sustain cell growth.

TAM accumulating in human and murine tumors show an IL10^{high} IL12^{low} phenotype and have very low tumoricidal activity consistent with an M2 biased phenotype. Sica *et al*, demonstrated that the defective IL-12 expression was due to autocrine production of IL-10 and was paralleled by the lack of NFkB activation, suggesting that the macrophage-production of immunosuppressive cytokines is part of the tumor strategies to escape immune surveillance (Sica *et al.*, 2000).

M2 macrophages produce a variety of factors that promote growth and invasiveness of tumor cells, including fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and epidermal growth factor (EGF). EGF for example, promotes tumor cell proliferation and

macrophages expressing EGF are often localized near blood vessels within the tumor suggesting that it may promotes blood extravasion of tumor cells (Pollard, 2004).

M1 macrophages are capable of tumoricidal activity through different molecules among which nitric oxide and TNF- α are the most important.

NO is produced from L-arginine by iNOS, an enzyme inducible upon exposure to inflammatory cytokines or bacterial products, NO mediates tumor suppression by inducing cell death via necrosis and/or apoptosis, therefore disrupting membranes and organelles such as mitochondria or inducing DNA fragmentation and cell shrinkage (Klimp et al., 2002). TNF- α was the first cytokine described to mediate the killing activity of macrophages (Carswell et al., 1975). Among the effects reported, there is the generation of reactive oxygen intermediates in mitochondria that results in cell membrane permeabilization (Goossens et al., 1995), and the induction of DNA-strands breaks (Baloch et al., 1990). Other members of the TNF family such as TRAIL and FasL provoke cell death but their role in macrophage-mediated tumor cell killing has not been elucidated (Klimp et al., 2002). Interestingly, TNF- α production by human cancer correlates with poor prognosis. The ability of TNF- α to promote angiogenesis, to induce chemokines and to stimulate fibroblast growth and functions, has been proposed to be involved in its pro-tumoral function (Balkwill, 2002). Thus TNF- α , has contrasting activities depending on many factors, among them its concentration.

Although macrophages seem capable of destroying tumor cells, tumor microenvironment potently halts such ability. For example phosphatidylserine, which is a natural component of tumor cell membrane, inhibits macrophage tumor cytotoxicity likely through inhibition of NO synthesis (Calderon et al., 1994). IL-4 and IL-10, alone or in combination, affect tumoricidal activity in part by the suppression of IL-1, TNF- α and IFN- γ synthesis (Nabioullin et al., 1994) (Oswald et al., 1992).

Because TAM can promote tumor growth but still maintain the intrinsic capacity to kill tumor cells, they could be a target of antitumor therapy. Possible strategies could aim at reducing their number and/or increasing their tumoricidal activity by switching their phenotype to M1. Factors that reduce the number of TAM or affect their functionality were tested for their ability to halt tumor growth. For example, Linomide was shown in mice to have the ability to reduce TAM number and to decrease tumor growth likely through impairing angiogenesis (Joseph and Isaacs, 1998).

Differently, Paclitaxel and Prolactin enhanced IL-12 production by mouse TAM and contributed to restore a Th1 response in tumour bearers (Majumder et al., 2002) (Mullins et al., 1999).

Chemokine inhibitors too, were shown to affect tumor growth by reducing macrophage infiltration (Robinson et al., 2003).

A number of studies demonstrated that IL-10 pushes macrophages towards a pro-tumoral phenotype, suggesting that IL-10 inhibition could restore the killing capacity of macrophages and activates type I antitumor immune responses (Sica et al., 2000). This latest approach has been investigated in this thesis.

Adoptive macrophage immunotherapy has been studied in a clinical setting [reviewed in ref. (Klimp et al., 2002)]. Macrophages were differentiated and activated from peripheral blood monocytes and reinfused into patient systemically. Although no clinical responses have been reported, biological response such as increase blood level of inflammatory cytokines IL-1, IL-6 and TNF- α were found in treated patients. The problem with this approach may be the poor recruitment of autologous activated macrophages at the tumour site, suggesting that additional studies are needed to assure a correct localization and activation of reinfused macrophages (Klimp et al., 2002).

1.5.3 Defective function of DC in cancer

In cancer patients, defective DC may result from abnormal myeloid-cell differentiation which has at least three main consequences: decreased number of circulating DC, accumulation within tumor of DC that are unable to upregulate costimulatory molecules or to produce cytokines necessary for T cell priming, and the increased production of immature myeloid cells with immune suppressive functions [reviewed in ref. (Gabrilovich, 2004)]. Early studies in animal models demonstrated a lower number of DC in LN, spleen and skin of tumor-bearing mice compared with controls animals (Gabrilovich et al., 1996). These studies were extended to human where a two/four fold decrease in the number of circulating DC was found in patients affected by head and neck cancer. Moreover clinical studies demonstrated that patients that had the primary tumor surgically removed showed restored number of circulating DC.

Because of decreased DC number, cancer patients are poorly responsive to immune stimulation (Gabrilovich, 2004).

Even more dramatic are the effects exerted by the tumor on TIDC (tumor infiltrating DC). A large number of studies demonstrated that DC infiltrating human tumors have an immature phenotype associated with poor activity. an observation that has been confirmed in several mouse tumor models [reviewed in ref. (Vicari et al., 2002)].

The first tumor-derived factor shown to inhibit DC function was VEGF. VEGF is produced by most tumors and has a crucial role in the recruitment and organization of tumor neovasculature. VEGF acts directly on the haematopoietic progenitors and inhibits DC maturation in vitro. Infusion of VEGF in mice resulted in inhibition of DC development thus confirming the in vitro results. The use of blocking Ab to VEGF abrogated the negative effect of tumor-cell-conditioned medium on DC differentiation in vitro (Gabrilovich et al., 1998; Ishida et al., 1998).

Another well-known immunosuppressive factor is IL-10, which is produced by the tumor itself or by its infiltrating leukocytes (Vicari et al., 2002). IL-10 suppresses the proinflammatory functions of DC by antagonizing expression of costimulatory molecules, the release of proinflammatory cytokines and DC maturation (Marincola et al., 2003). In mouse, addition of recombinant IL-10 to splenic DC halted their ability to secrete IL-12 and to prime T cells in vivo (De Smedt et al., 1997). Interestingly, Faulkner *et al*, demonstrated that IL-10 does not block phagocytic activity of DC but their maturation and consequently T cell activation (Faulkner et al., 2000).

Tumor-induced IL-10 was found to be specifically responsible for DC dysfunctions in response to antigenic driven maturation. Splenic DC from tumor bearing mice were found to be defective in stimulating allogenic proliferation and tumor-antigen specific T cell responses. Authors showed that in absence of IL-10, DC from tumor bearing mice were equally effective in stimulating type-1 immune response to non-tumor bearing mice and that the lack of IL-10 resulted in tumor rejection (Yang and Lattime, 2003).

Another negative effect of IL-10 in the tumor milieu is to prevent DC accumulation at tumor site. Qin *et al*, analyzed the effect of spontaneous IL-10 expression by the J558L mouse plasmacytoma. J558L cells engineered to express GM-CSF were ineffective as vaccine when used to prevent growth of the parental cell line. In the presence of the IL-10, DC accumulation promoted by GM-CSF was completely inhibited rendering the vaccine ineffective. Reversion of this phenotype was observed upon block of IL-10 (Qin et al., 1997).

Similarly to IL-10, TGF- β has been shown to have a wide range of immunosuppressive effects on DC, including down-regulation of cell surface MHC, costimulatory molecules and chemokine receptors. In particular Kobie *et al*, demonstrated that TGF- β halts the capacity of TIDC to reach DLN and prime T cells. Combinatory use of antisense TGF- β

gene transfer plus TGF- β -neutralizing antibody, increased the efficacy of DC vaccines in treating established TGF- β -secreting 4T1 mammary carcinoma (Kobie et al., 2003).

The above observations suggest that the tumor microenvironment not only does not provide activatory stimuli to TIDC, but inhibits their differentiation and/or functions. DC inactivation occurs also systemically (Vicari et al., 2002). In particular the systemic effects have to be taken into account when planning immunotherapeutic approaches that use ex-vivo loaded DC to be reinfused into cancer patients to generate T cell response. Enk *et al*, demonstrated that TIDC derived from progressive melanoma tumors have decreased ability to stimulate T cell proliferation in an allogenic setting and moreover induced tumor specific T cell tolerance. Thus tumor-derived factors can convert DC antigen presenting function to induction of tolerance against tumor TAA rendering tumor DC active "silencers" of anti-tumoral immune responses (Enk et al., 1997).

These observations should be taken into account in all the approaches that foresee DC injection into the tumor or the use chemokines to attract DC at the source of tumor antigens. In addition to fail, these approaches are likely to worsen the host immune response generating tolerance to TAA.

A model of the possible alternative functions of tumor infiltrating macrophages and dendritic cells is given in Figure 1.6.

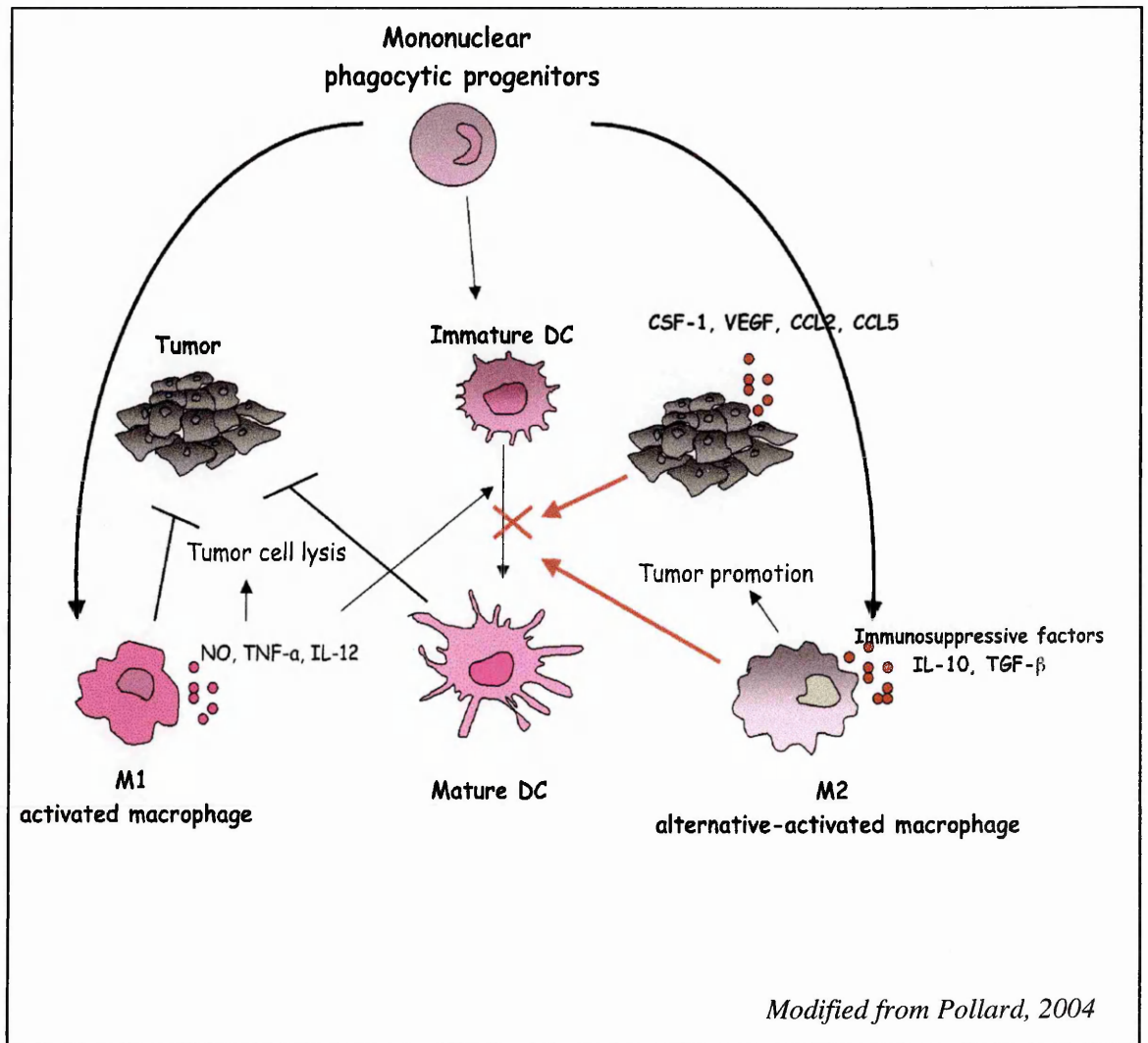


Figure 1.6 Pro- and anti-tumorigenic properties of macrophages and DC infiltrating tumor.

1.6 Aims of the thesis

Many protocols of cancer immunotherapy have been designed to induce tumor-specific CTL that are thought to be the most efficient effector cells to destroy existing tumors. Based on the critical role of DC in inducing T-cell dependent immunity, significant effort has been placed in studies based on the use of TAA-loaded DC to be used as vaccine or in strategies that, by enriching DC at tumor site, are expected to promote a tumor specific CTL response. However, when translated in clinical trials many of these approaches were found to induce a weak response that inversely correlates with tumor size.

Likely, the presence of systemic and local induced-immune suppression halts the effectiveness of such approaches. Combinatory treatments able to simultaneously break tumor induce immunosuppression and to load DC with TAA may then result in a more effective therapy for established tumors.

The aims of this thesis were:

- To characterize the antitumor activity of the human chemokine CCL16 in murine models of prestablished tumors;
- To study whether CCL16 can block the metastatic spreading of 4T1 mammary carcinoma, a model of spontaneous metastases from primary tumor;
- To determine the effect of the tumor microenvironment on leukocytes recruited by CCL16 at the tumor site;
- To study whether block of the IL10R and stimulation with microbial stimuli, CpG, could break the infiltrate paralysis;
- To characterize the synergy between a strong innate response, triggered at tumor site, and the resulting systemic adaptive response, in the rejection of large tumors.

CHAPTER 2. MATERIALS AND METHODS

2.1 Tumor cells and mice

The murine colon adenocarcinoma cell line C26 was derived from BALB/c mice treated with N-nitroso-N-methylurethane (Griswold and Corbett, 1975). TSA tumor is a murine mammary adenocarcinoma that arose spontaneously in a multiparous BALB/c mouse (Nanni et al., 1983). 4T1 tumor is a 6-thioguanine-resistant cell line derived from a spontaneous mammary carcinoma (American Tissue Culture Collection, ATCC-LGC Promochen, United Kingdom) (Aslakson and Miller, 1992). MCA38 (H2^b) is a colon carcinoma cell line established from primary cultures (Goldrosen et al., 1976). 293 are human embryo kidney cells transformed by shared Adenovirus type 5 DNA, used to complement the growth of E1 defective Adenoviral vectors (Harrison et al., 1977). Murine tumor cells were cultured in DMEM supplemented with 10% FCS, 200U/ml penicillin, 200 µg/ml of streptomycin and 600 µg/ml of L-glutamine (Biowhittaker, Walkersville, MD). 293 were cultured in EMEM (Biowhittaker) supplemented as above described.

Eight-10 wk old female BALB/c mice, BALB/c nu/nu mice and C57/BL6 mice were purchased from Charles River (Calco, Italy).

IL-12p35-KO and IFN- γ KO mice (GKO) mice on BALB/c background, TNF-KO mice on C57/BL6 background and B6.SJL/J (CD45.1) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA).

CD40-KO mice, backcrossed for 10 generation to BALB/c, were kindly provided by L. Adorini (Roche, Milan, Italy) (Castigli et al., 1994). CCR7-KO mice were kindly provided by M. Lipp (Max-Delbruck-Center, Berlin). All mice were maintained at the Istituto Nazionale Tumori under standard conditions according to institutional guidelines.

2.2 Construction of recombinant adenoviral vector.

Recombinant adenovirus carrying the human CCL16 gene under the control of mouse CMV promoter was constructed using the Cre-lox recombination system (Microbix, Toronto Canada) (Ng P, 2000). The 500 bp fragment containing the cDNA of CCL16, derived from PCDNA3/CCL16 (a kind gift of Prof. Mirella Giovarelli, University of Turin, Turin, Italy) was excised with *Eco RI* and cloned into *Eco RI* site of pDC316 adenoviral shuttle plasmid, which is designed for the insertion of coding sequences for rescue into vectors by Cre-mediated recombination (see chapter 3.1.1 for maps of the plasmids). PDC316/CCL16 plasmid was sequenced before use to verify the correct sequence of the inserted CCL16 cDNA.

Recombinant adenovirus were isolated from a single plaque amplified on 293 cell monolayers and purified by double cesium chloride gradient centrifugation as below described according to Hitt *et al.* (Hitt, 1998). Experiments were carried out with at least 5 independently prepared adenovirus stocks. The empty adenoviral vector Add170-3 was kindly provided by Dr. Frank Graham (Mc Master University, Hamilton, Canada) (see figure 2.1 for scheme of the steps involved in rescue of Ad vectors).

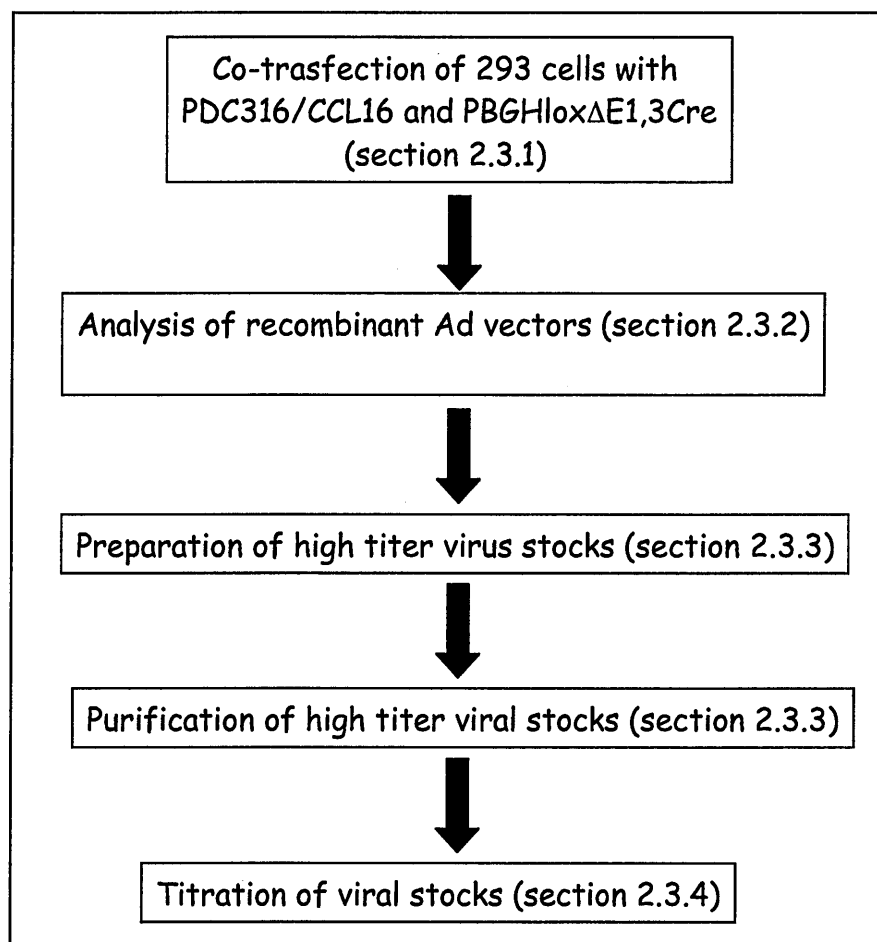


Figure 2.1: Overview of the steps involved in rescue, propagation, purification of AdCCL16.

2.2.1 Cotransfection of 293 cell line

PDC316 shuttle plasmid containing CCL16 cDNA was cotransfected together with PBHGloxΔE1,3Cre genomic plasmid, in 293 cells. The cotransfection was performed in 60mm dishes containing 70-80% confluent, low passage 293 cells (passage 27-38) using calcium phosphate method. For each 60mm dish 10μg of salmon sperm DNA was added to 0.5 ml sterile 1x HeBS (21mM HEPES, 137 mM NaCl, 5mM KCl, 0.5 mM Na₂HPO₄, 5.6 mM glucose, adjusted to PH 7.1) and the DNA was sheared by vortexing 1 min. Five micrograms of the shuttle plasmid PDC316/CCL16 and of the genome plasmid

PBHGloxΔE1,3 Cre was added to the mix and mixed gently. Following this, 25 µl of 2.5 M CaCl₂ was added for 0.5 ml of DNA solution and after incubation at RT for 30 min the precipitate was added to 293 cells. Dishes were incubated with the DNA precipitate for 4-5 h at 37°C. After incubation the medium was removed and cells were overlayed with 1:5 mixture of overlay medium (EMEM supplemented with 5% inactivated horse serum, 200U/ml penicillin, 200 µg/ml of streptomycin and 600 µg/ml of L-glutamine) to 1% agarose. Dishes were incubated at 37°C until plaques developed (5-10 days).

2.2.2 Screening of recombinant viral plaques

An agar plug containing the viral plaque was removed from the dish the stored at -80°C in 0.5 ml of sterile PBS (137 mM NaCl, 3 mM KCl, 4 mM Na₂HPO₄) containing 10% glycerol. In order to screen plaques, 0.2 ml of the plaque isolate was used to infect a 60mm dish of 293 cells. Following incubation for 45 min at 37°C, 5 ml of complete medium was replaced and when the complete cytopathic effect (CPE) was observed (90% of the cells no longer attached to the dish) the cells were allowed to settle for 30 min at RT and the supernatant removed. One ml of the viral supernatant was stored at -20°C and used to detect the presence of secreted CCL16 by ELISA and part was added to glycerol (10% final concentration) and stored at -80°C. The remaining cells on the dish were lysed in 0.5 ml pronase-SDS (500 µg of pronase in 10mM Tris pH 7.4, 10 mM EDTA, 0.5% SDS). The DNA was then extracted by addition of 1/10 of 3M sodium acetate and 2 volume of cold ethanol. After centrifugation at 16000 rpm the DNA pellets were washed twice with 70% ethanol dried at RT and resuspended in water. The structure of the viral DNA was analysed by restriction enzyme digestion.

2.2.3 Propagation and cesium chloride banding of the virus

293 cells were seeded in 150mm cell culture dishes and when confluent used to prepare high titer-stocks. Four ml of viral screening supernatant was diluted to 36 ml of medium and used to infect a total of eighteen dishes (2 ml for dish). Infection was carried out for 45 min at 37 °C after which 25 ml of complete medium was added. After complete CPE the infected cells were collected by centrifugation and resuspended in 5 ml of PBS containing 10% glycerol and stored at -80°C. Virus was titrated prior to further use (see the following section). 293NS cells were used for the preparation of cesium chloride gradient purified viral stocks. 293NS are a variant of 293 cells able growing in suspension in Pro-293-CDM medium (Biowhittaker) supplemented with 5% of foetal calf serum. Three litres of 293NS spinner culture (at a density of 4×10^5 /ml) was centrifuged at 2000 rpm for 15 min, half of the conditioned medium was saved and the cell pellet was resuspended in 100 ml of fresh medium containing 5 PFU of crude viral stock. Cells were incubated at 37°C, with stirring, for 2 hr, at which time the infected cells were transferred back to the spinner flask along with the reserved conditioned medium and an equal volume of fresh medium. After 3 days, cells were harvested by centrifugation and pellet resuspended in 30 ml of 10mM Tris HCl and stored at -80°C until cesium chloride gradient banding.

The frozen infected cells suspension was thawed and 1/10 volume of 5% sodium deoxycholate was added. The solution was incubated at RT for 30 min after which DNase I (bovine pancreatic deoxyribonuclease I, Sigma-Aldrich) was added at a final concentration of 1mg/ml. The solution was incubated at 37°C for 30min, centrifuged at 3000 rpm for 20 minutes and 5 ml of it applied on the top of a CsCl gradient prepared as followed: at 13 ml ultraclear tube was added 0.5 ml of 1.5 g/ml solution, followed by 3 ml of 1.35 g/ml and by 2.5 ml of 1.25 g/ml. Gradients were centrifuged at 35000 rpm for 1 hr in a Beckman SW 41T1 rotor. The virus band, visible as a whitish band in the upper 1/3 of the tube, was collected by puncturing the tube, and transferred into a 5 ml ultraclear tube, topped with

1.35 g/ml CsCl solution, mixed and centrifuged for 16 hr at 35000 rpm in a Beckman SW 55T1 rotor. The viral band was collected again by puncturing and then dialyzed twice against 1 litre of 10mM TrisHCl pH 8, for at least 4 hr per dialysis medium change.

2.2.4 Titration of viral stocks

Virus titer was determined by plaque assay in 293 cells and given as Plaques Forming Units (PFUs). Briefly virus stocks were serially diluted in PBS and 0.25 ml of each dilution was used to infect 60 mm dishes of 293 cells. Following infection the cells were overlaid with agarose containing medium as previously described. Plaques were counted 7 days post-infection and the titer was calculated as follows: number of plaque \times 4 (dilution factor) = pfu/ml.

2.3 Adenovirus infection of tumor cell lines in vitro

In vitro infections were carried out as follows: 5×10^5 TSA or C26 cells were plated in 60 mm cell culture dishes. After 10 h cells were infected with various MOI of AdCCL16 or Add170-3 virus. Supernatants were collected 24 and 48 h later and the amount of CCL16 secreted was quantified by ELISA.

2.4 In vitro differentiation of DC from bone marrow

DC were differentiated from bone marrow as follows: bone marrow cells from femurs and tibias were plated at 5×10^5 /ml in RPMI 5% FCS supplemented with 20% of a murine fibroblast cell line engineered to express mGM-CSF (corresponding to 20 ng/ml of GM-CSF). On day 2, one-half of supplemented medium was replaced with fresh medium. On day 4, floating cells were centrifuged and plated in fresh medium at 5×10^5 /ml. On day 5 non-adherent cells were harvested. DC collected on day 5 were 90% CD11c positive and

displayed the following characteristic phenotype: MHC classII^{intermediate}, CD80^{low}, CD40^{low}, CD86^{low}. In some experiments BM-DC were matured by seeding them at a concentration of 2×10^6 /ml in the presences of 1 μ g/ml of LPS (Sigma-Aldrich) for 10h.

2.5 Chemotaxis assay

CD4 and CD8 lymphocytes were obtained from naïve mouse spleens; the two populations were enriched more than 95% by positive selection using MiniMacsTM separation columns (Miltenyi Biotec, Germany). Immature and mature DC were obtained as above described. Recombinant human CCL16 and mouse CCL20 were purchased by PeproTech (Rocky Hill, NJ). Chemotactic activity was tested using the Boyden chamber method as described (Zigmond and Hirsch, 1973). Briefly, triplicate assay were carried out in 96-well microchemotaxis chambers (Neuro Probe, Cabin John, MA) with an 5- μ m pore polycarbonate membrane (Costar, Cambridge, MA) separating cells from the medium containing the chemoattractant. T cells (4×10^6 /ml) or DC (2×10^6 /ml) were allowed to migrate for 3 or 2 hr respectively, in DMEM medium containing 0.1% of BSA. When necessary cells, were preincubated with 200 ng/ml pertussin toxin (Sigma Chemical Co., St. Louis, MO) for 1 hr at 37 C⁰. The membrane was removed and stained with Diff Quick kit. Migrated cells were counted with a Nikon microscope (40X objective).

In some experiments, viral supernatants were used as the source of CCL16. To obtain these supernatants TSA cells were plated at a concentration of 2×10^5 /ml, 1 day later infected with 100 MOI of AdCCL16 or Addl70-3, and supernatants harvested 48 hr later. CCL16 in the medium was quantified by ELISA.

Migration index was calculated as: number of cells migrated in the presence of recombinant or viral CCL16/number of cells migrating in medium alone or in supernatant of Addl70-3-infected TSA cells, respectively.

2.6 Tumors injection and in vivo treatments

BALB/c mice were inoculated s.c. into the right flank, equidistant from the inguinal and axillary lymph nodes, with 5×10^4 C26 or 1×10^5 TSA or 7×10^3 4T1 cells. After 7-10 days, when tumors reach 3-4 mm in diameter (depending on the experiment), nodules were inoculated with 1×10^9 pfu of AdCCL16 or control virus Addl70-3 and the treatment was repeated four days later or not as indicated.

For the part of the project in which the synergy between AdCCL16 and CpG plus antiIL10R treatment was studied, 2×10^5 TSA or 1×10^5 MCA38 or 1×10^4 4T1 cells was inoculated s.c. into the right flank of the mice. After 10-14 days, when tumors reach 5 mm in diameter, nodules were inoculated with 1×10^9 pfu of AdCCL16 or control virus Addl70-3 and 36 h later with 5 μ g of CpG 1668 (5'-TCCATGAGGTTTCCTGATGCT-3') (MWG, Germany) while 200 μ g anti-IL10R antibody (1B1.3a; IgG1) or matching control rat IgG 1 antibody (clone GL113) (Vicari et al., 2002) were given intraperitoneally. Purified anti-IL10R and control antibodies were provided by our collaborator Dr. Alain Vicari (Schering-Plough, Dardilly, France).

In some experiments, mice were depleted of CD4 and CD8 cells by i.p. injection of 250 μ g/mice of anti-CD4 (GK1.5 hybridoma, Lyt2; ATCC) or anti-CD8 (clone 2.43; ATCC) mAb. Depletion was started one week before tumor challenge by injecting mice twice per week thereafter until the end of the experiment. In some experiments, depletion was started five days after tumor treatments. FACS analysis of peripheral blood confirmed that depletion was never inferior to 95%.

For surgical excision of primary 4T1 tumors, mice were anesthetized before surgery and tumors resected with sterilized instruments. Wounds were closed with metallic clips. All mice survived surgery. Mice in which primary tumors recurred at the site of the surgical excision (4 out of 60) were eliminated from the experiment.

Tumor growth and size were recorded twice each week and tumor growth was expressed as % of tumor free mice over total injected mice, while tumor size was measured with calipers, and volume was calculated by the formula: $Vol = (\text{longest diameter}) \times (\text{shortest diameter})^2$ and expressed in mm^3 .

2.6.1 Quantification of 4T1 distant metastases

Because 4T1 are 6-thioguanine-resistant 4T1 metastatic cells can be detected and quantified by explanting organs, plating dissociated cells in medium supplemented with the drug (Pulaski and Ostrand-Rosenberg, 1998; Pulaski, 2000). Briefly lungs were collected and dissociated in a HBSS solution containing 1mg/ml collagenase type 4 and 6 units/ml elastase for 1 h at 4 °C; organs were then plated either neat or serially diluted in 10 cm cell culture dishes in a medium supplemented with 6-thioguanine (10ng/ml). Individual colonies representing micrometastasis were counted after 10 –15 days. Colonies were counted as follows: medium was discarded and 5 ml of methanol was added to each plate and incubated for 5 min at RT, washed with PBS and stained with GIEMSA staining solution (Sigma-Aldrich, St. Louis, MO) according to manufacture instructions.

2.7 Morphological analysis and immunocytochemistry

Tumor fragments and tumor-draining lymph nodes were embedded in OCT compound (Miles Laboratories, Inc., Elkhart, Nederland), snap-frozen in liquid nitrogen, and stored at -80°C. For histological evaluation, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin or Masson's trichrome according to manufacture instructions (Sigma-Aldrich).

For immunohistochemistry, acetone-fixed 5 μm cryostat sections were incubated 5 min in 3% MeOH/H₂O₂ to block endogenous peroxidase. After washing for 10 min in PBS,

sections were incubated 1h with the following antibodies: anti-CD11c (clone N418, Chemicon International, Temecula CA), anti-CD11b/CD18 (clone M1/70.5), anti-CD8 (Ly/T2, clone YT5 169.4) and anti-CD4 (LT34, clone YT5.191.1.2) (all from Sera-lab, Crawley Down, Sussex, UK), or anti-GR1 (clone RB6-8C5, ATCC), anti-NK (asialo GM1)(Wako Chemicals GM&H, Dusseldorf, Germany), anti-F480 (clone C1:A31, Caltag) anti-IFN- γ (clone XMG1.2, provided by Dr. S. Landolfo, University of Turin, Turin, Italy). After washing, sections were overlaid with biotinylated goat anti-rat, anti-hamster and anti-rabbit, and horse anti-goat Ig (all from Vector Laboratories, Burlingame, CA) for 30 min. Unbound Ig was removed by washing and slides were incubated with Avidin-peroxidase complex (Dako, Glostrup, Denmark). Sections were then incubated with 0.03% H₂O₂ and 0.06% 3,3'-diaminobenzidine (Sigma-Aldrich) for 2-5 min, washed in tap water and counterstained with Mayer's hematoxylin (Sigma-Aldrich), dehydrated in graded alcohol (70, 95 and 100% ethanol) and mounted in BDH medium (Merck Eurolabs) according to the manufacturer's instructions. Tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used as secondary antibody for CD8+ cell immunofluorescence and fluorescein (FITC)-conjugated chicken anti-goat IgG (Rockland Inc., Gilbertsville, PA) used as secondary antibody for IFN- γ immunofluorescence.

For the intracellular staining of IL-10, IL-12 and TNF- α cytokines, 5 μ m-cryostat sections were fixed with 2% PFA for 20 min and washed. Sections were sequentially incubated for 30 min with the following solutions made in PBS: 1) 1% BSA 2) 0.1% saponine /1% FCS. After washing, sections were incubated with avidin-biotin blocking solution (Vector) for 15 min. After washing, sections were incubated with the following monoclonal antibodies: a) rat anti-mouse IL-10 (clone JES5-2A5, eBioscience), rat anti-mouse TNF- α (clone XT22, BD Bioscience), and rat anti-mouse IL-12p70 (clone 9A5, Endogene). One-hour

later, sections were washed and then incubated with secondary antibodies and revealed as above described.

Cell counts were obtained in 10 randomly chosen fields on a 1mm² grid and is given as cell/mm² (mean \pm SD). Image were acquired with a light microscope (Nikon) equipped with a digital camera (DXM 1200, Nikon) and analyzed using ACT1 software (Nikon). Confocal microscopy was performed with Zeiss LSM 510 META.

Immunoistochemistry analysis was performed with the collaboration of Dr. Emma di Carlo (Department of Oncology and Neuroscience, University of Chieti, Chieti, Italy) and Dr. Sabina Sangaletti (Immunotherapy and Gene Therapy Unit, Istituto Nazionale Tumori, Milan, Italy).

2.8 Lymph node analysis

At the indicated time points, inguinal and axillary lymph nodes draining the treated tumors were harvested, mechanically disrupted and digested for 45 min at 37 °C with a solution of 1 mg/ml collagenase D (La Roche, Nutley, NJ) in HBSS (Biowhittaker). Lymph nodes were passed over a 70- μ m nylon filter and cells were counted and used for FACS analysis. All antibodies used were purchased from (BD Bioscience, San Diego, CA). For analysis of T cell subsets and DC populations, cells were stained with PE-conjugated anti-CD11c, anti-CD4 and anti-CD8. Analyses were performed on a FACScan (BD Bioscience). When indicated, lymph node cells were suspended at 1x10⁶ cells/ml and stimulated for 10 h with antiCD3 antibody (1 μ g/ml; PharMingen). Supernatants were tested for IFN- γ production by ELISA using specific antibodies (see below for details). Cells were analyzed for IFN- γ production in the CD4 and CD8 subsets using mouse IFN- γ secretion assay kit according to manufacture instructions (Miltenyi Biotec, Germany). Stained cells were analyzed by FACS analysis.

2.9 Analysis of tumor cell infiltrate by FACS analysis

For analysis of T cell infiltration, treated tumors were measured, harvested, removed of extraneous tissue, and digested for 1 h at 37°C in 1 mg/ml collagenase D (La Roche) in HBSS (BioWhittaker/Cambrex). Digested tumors were passed over a 70 µm nylon filter and washed once with RPMI complete medium. Each smashed tumors were resuspended in 3 ml of RPMI complete medium and let to adhere on 35 mm diameter dishes for 4 hr after which the floating leukocyte infiltrate was collected and counted. For analysis of T cell subsets and DC populations, cells were stained with PE-conjugated anti-CD11c, anti-CD4 and anti-CD8. All antibodies used were purchased from (BD Bioscience, San Diego, CA). Analyses were performed on a FACScan (BD Bioscience) with counting of 50000 events. The number of infiltrating CD4 or CD8 or DC were expressed as number of cells/mm³ and calculated as follows: percentage of positive cells x total number of infiltrating leukocytes x100/tumor volume. The data were normalized to the tumor volume, because the tumors were of different sizes.

2.10 IFN-γ production by env-specific CTL clones

Six mice bearing TSA tumors were treated with AdCCL16 on days 0 and 4. Two days after the last treatment, mice were divided into two groups. DLNs from each group were pooled and treated independently. DC were enriched from the lymph node populations using anti-CD11c magnetic beads (Miltenyi Biotec, Germany) and used to stimulate a specific CTL clone. The negative fraction of DC purification containing lymphocytes was used to test CTL activity (see below). The generation and maintenance of the CTL line E-88 specific for murine leukemia virus (MuLV) env-derived peptide 423-431 SPSYVYHQF (AH1) has been described (Rodolfo et al., 1991). E-88 cells (10⁵ well of 96 well plates) were incubated with stimulating cells (2x10⁵ DC or 10⁵ TSA cells) for 18 hr at 37 C⁰ in 200 µl of complete medium (RPMI, supplemented with 10% FCS). Supernatants were tested for

IFN- γ content by specific ELISA (as described below). Lymphocytes from the CD11c-negative fraction were restimulated *in vitro* in a mixed lymphocyte tumor culture and tested for CTL activity as above described. TSA and C26 (sharing the *env* antigen with TSA) were the specific targets and blast cells, pulsed or not with AH1 peptide, were the control for TSA tumor-specific lysis.

2.11 Mixed lymphocyte tumor culture and cell-mediated cytotoxicity assay

Splenocytes (5×10^5 cells/ml) from treated mice were restimulated *in vitro* with the H2-L^d peptide AH1 (SPSYVYHQF) or H2-K^b KSP (1 μ g/ml) or with irradiated parental tumor cells (1 to 10 ratio tumor cells/splenocytes) in RPMI 1640 medium supplemented with 10% FCS and 10U/ml of hIL-2. The *env* gene of the endogenous ecotropic MuLV, containing the L^d-restricted peptide AH1 or the L^b-restricted peptide KSP, is expressed by TSA and MCA38 tumor as an immunodominant antigen (Huang et al., 1996). After 5 days, cytotoxic activity was tested in a standard 4-h ⁵¹Cr release assay. Cells were washed three times and resuspended in complete RPMI-10 medium at 2×10^4 /ml. 100 μ l of effector activated-splenocytes (2×10^6 /ml) were plated in U-bottom 96-well plates (Costar) with 100 μ l of ⁵¹Cr-labelled target cells at different E:T ratios starting from 100:1 (100 μ l of 2×10^4 /ml target cells). After a brief centrifugation at 500 rpm, the plates were incubated for 4 h at 37°C. The plates were then centrifuged for 5 min at 500 rpm, 80 μ l of supernatants were harvested into 96 well LumaPlates (Packard, Groningen, Netherlands) and the radioactivity of the supernatants samples was determined using a γ - irradiation counter (Top count microplate scintillation counter, Packard). The percent of lysis was calculated using the following equation:

$$\% \text{ lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100$$

where experimental cpm = cpm of supernatants from wells containing effectors plus targets, spontaneous cpm = cpm of supernatants from wells containing targets plus medium, and maximum cpm = supernatants of wells containing target plus TritonX-100.

Blast cells, pulsed or not with the env peptides, as well as tumor cells were used as specific targets.

2.12 In vitro and in vivo assay with tumor infiltrating DC (TIDC)

TIDC were purified from treated tumors as previously described (Chiodoni et al., 1999). In brief, tumor masses were perfused with collagenase D solution (1mg/ml) and incubated for 1 h at 37°C. After gentle pipeting, the suspension was allowed to adhere for 4 h at 37°C in complete RPMI medium after which non-adherent cells were collected and purified using CD11c⁺ microbeads (Miltenyi Biotech). Flow cytometry showed that the cells were more than 80% pure. Phenotypic characterization of TIDC was performed by FACS analysis using FITC-conjugated monoclonal antibodies: anti-CD40-FITC clone 3/23; anti-CD80 clone IG10; anti-CD86 clone GL1; anti-MHC-II clone B21.2 and isotype-matched monoclonal antibodies of unrelated specificity (BD Bioscience). TIDC were seeded in round-bottom non-coated 96-well plates at a concentration of 1x10⁶/ml in RPMI complete medium containing 5 ng/ml of GM-CSF (Peprotech). Supernatants were collected 48 hr later and assayed by ELISA for IL-12 using specific antibodies (as below described).

Experiments in which TIDC were used as T cell lymphocyte stimulators were performed as follows: CD4⁺ T lymphocytes were purified from spleen of C57/BL6 mice using CD4 microbeads (Miltenyi Biotech). Triplicate of 2x10⁵ CD4 T cells were seeded in round-

bottom non-coated 96-well plates with various ratios of irradiated (3000 rad) TIDC for 5 days. [^3H] thymidine (1Ci/well; Amersham) was added for the last 10 h of culture.

For *in vivo* DC-migration experiments, mice bearing TSA tumors were intratumorally injected with the indicated treatment plus 2×10^7 FITC-conjugated latex particles of 1 μm of diameter (Polysciences, Warrington, PA). At the indicated time points, mice were sacrificed and DLN were collected, teased and incubated for 1 h in collagenase D solution (1mg/ml). Cells were stained for CD11c expression and 5×10^5 events per LN were acquired by cytofluorimetry. The total number of double positive CD11c-PE/beads-FITC events was calculated for each LN.

2.13 Nitric Oxide evaluation

Total NO in tumor samples was determined as function of nitrate and nitrite concentrations using a specific kit (nitrate and nitrite colorimetric assay kit, Cayman Ann Arbor, MI). In brief, 5 mm tumors were treated with the various combinations, collected 4 h later, perfused and minced in 300 μl of PBS. The homogenate was centrifuged at 10000 rpm for 20 min and then ultra filtered using a 30 KDa molecular weight cut-off (Millipore). Forty μl of each filtrate was assayed for nitrate and nitrite concentration according to the manufacturer's instructions.

2.14 Bone marrow chimera

CB6F1 mice were lethally irradiated with 900 cGy (given as a split dose 450+450 cGy at a 3 h interval). Two hour later, mice were injected i.v. with 2×10^7 bone marrow (BM) nucleated cells obtained from BALB/c or C57L/B6 mice. BM cells were obtained by flushing the cavity of freshly dissected femurs with complete RPMI medium. To verify engraftment, PBMCs withdrawn from the retro-orbital sinus at 8 weeks after BM

transplantation were stained with FITC-conjugated anti-mouse H2K^b and PE-conjugated anti mouse H2K^d as well as matching isotype controls.

B6.SJL mice were reconstituted with BM from TNF-KO mice to obtain mice lacking TNF- α in the BM compartment. Engraftment was verified using CD45.2 Ab (BD Bioscience). We used mice showing at least 95% of donor phenotype.

2.15 Enzyme-linked immunosorbent assay (ELISA): CCL16, CCL2, IFN- γ , IL-12 and TNF- α measurement

To perform the ELISA, BD Bioscience IL-12 (clone 9A5 as capture Ab and C17-8 as detection antibody), TNF- α (clone XT22 as capture Ab and XT3 as detection antibody), IFN- γ (clone R4-6A2 as capture Ab and XMG1.2 as detection Ab) or Peprotech Abs detecting CCL16 or CCL2 were used following the standard procedure. Briefly, anti-cytokines capture Abs were diluted to 2 μ g/ml in coating solution (0.1 M NaHCO₃, pH 8.2). 50 μ l of diluted Ab was added to wells of an enhanced protein-binding ELISA plate (Nunc Maxisorb, Life technologies, Naperville, IL) and incubated overnight at 4°C. Capture Ab solution was removed and non-specific binding was prevented by adding 200 μ l/well of blocking buffer (1X PBS/10% fetal calf serum). The plate was incubated 1 h at 37°C and washed 3 times with PBS/Tween (0.05% Tween-20 in PBS). 50 μ l/well of samples and a serial dilution of a standard cytokine protein solution of known concentration was added and left for 2-4 h at 37°C. The plate was washed 4 times with PBS/Tween, 50 μ l per well of diluted biotinylated anti-cytokine detection Ab (1 μ g/ml) was added and incubated 1 h at RT. After washing 4 times with PBS/Tween, 50 μ l/well of diluted Avidin-Horseradish Peroxidase (e.g. Sigma-Aldrich 1:400 of 1 mg/ml solution) was added and plates were incubated for 30 min at RT. After washing 8 times with PBS/Tween. 100 μ l/well of 2,2'-Azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) substrate solution (150 mg ABTS to 500 ml of 0.1 M anhydrous citric acid in dH₂O, pH

4.35; 10 ml/11ml of 30% H_2O_2 added prior to use) were used for color development and the optical density (O.D.) for each well was read with a microplate reader (MRX microplate reader, DYNEX) set to 405 nm.

2.16 Statistical analysis

Data were analyzed using a two-sided Student's t-test, except for metastasis formation data, which were analyzed using the Mann-Whitney test. All analysis were performed using Prism software (GraphPad Software). Differences were considered significant at $p < 0,05$.

CHAPTER 3: GENERATION OF ADENOVIRAL VECTOR EXPRESSING CCL16 AND CHARACTERIZATION OF VIRAL AND RECOMBINANT CHEMOKINE.

3.1 Introduction

Vectors based on adenovirus have been widely used in cancer gene therapy because of their capacity to infect efficiently a wide variety of cell types. This virus has been well characterized throughout the last 30 years and has been chosen as vector backbone because it is relatively non-pathogenic producing symptoms, at worse similar to influenza. Unlike retroviruses, adenoviruses can infect both quiescent and proliferating cells and do not integrate into the host genome, a characteristic that confers important safety feature for in vivo gene transfer [reviewed in ref. (Hitt and Graham, 2000; Mizuguchi et al., 2001)].

Although adenoviruses have been shown to induce oncogenic transformation of rodent cells, no serotypes have ever been related to human cancer or any naturally occurring malignancy in other species.

Currently, adenovirus vectors are derived mainly from serotypes 2 and 5 - the most common serotypes to which humans have been exposed. After virus entry, the vector borne transgene, is expressed at very high level but only transiently, vanishing after about two weeks.

To accommodate large amounts of DNA a number of manipulations have been performed leading to the development of three types of adenovirus vectors, namely first, second and third generation vectors.

First generation vectors were obtained by deleting the E1 and/or E3 regions of the genome. This deletion abolishes the transforming potential of the virus, renders the virus replication defective and allows insertion of a larger amount of foreign DNA (up to 8 kb). Propagation

of these vectors requires complementing cell lines that provide in *trans* the products of the E1 region necessary for the initiation of viral replication.

Many techniques have been developed to construct first generation adenovectors. In earlier methods, foreign DNA was inserted into a plasmid containing the left end of the Ad genome, the inverted terminal repeat (ITR), the gene of interest, and this was joined with a plasmid with all the viral genome modified to be non infectious. The ligation was done either in vitro with restriction enzymes or in vivo by homologous recombination between two overlapping sequences in 293 cells (Mizuguchi et al., 2001).

Ng and coworkers substituted homologous recombination with Cre-mediated recombination, further improving the efficiency and the accuracy of the process (Ng P, 2000). The major problem with first generation adenovectors is their tendency to induce immune response against capsid proteins. This is a major disadvantage when, as in the case of gene therapy of hereditary disease, long-term expression of the transgene is needed, but may be advantageous in cancer treatment where often there is no need for long-term expression and where the virus immunogenicity may enhance the clearing of infected tumor cells. Subsequent manipulations of the viral genome were designed to decrease vector immunogenicity throughout deletions of additional early genes. Recently, a third generation of adenovectors, the helper-dependent or gutless vectors, were generated. In this system, deletion of all viral sequences, except for the packaging signal, increases the cloning capacity up to 37 kb and significantly decreases the virus immunogenicity. This renders the vectors more attractive for gene therapy of somatic disorders. However, their isolation and purification are time consuming and need to be simplified for a scale up compatible with pharmaceutical distribution (Mizuguchi et al., 2001).

First generation adenoviral vector have been widely used in cancer immunotherapy. The major advantages of these vectors are: 1) a broad host range in terms of target tissue; 2)

easy preparation of highly purified viral stocks for in vivo treatments; 3) an high cloning capacity; 4) a transient expression that may limits the toxicity of the active molecule.

A number of studies in animal models demonstrated that the presence of neutralizing antibodies might limit the effectiveness of Ad-mediated gene transfer in vivo. This has posed major concern in the use of adenovector for cancer therapy since the majority of the population has been exposed to this virus during their lifetime. Most of the studies done have focused on administration of Ads into tissue such as liver or lung which being in direct contact with mucosal surfaces are more susceptible to anti-Ad antibodies. On the contrary solid tumors may be less susceptible to the penetration of anti-Ad antibodies. Bramson *et al.*, investigated this issue and found that in animal pre-immunized with adenovirus the outcome of Ad-IL-12 delivery, in terms of transgene expression and effectiveness, was not different from that of non-immune mice (Bramson et al., 1997). Moreover they demonstrated that pre-existing immunity limits the spreading of the virus to distant organs such as liver without affecting transgene expression at tumor site.

Anti-tumor activity and induction of systemic immunity, in animal models, have been reported for adenoviruses expressing a variety of cytokines and chemokines, for example: IL-2, IL-12, TNF- α , GM-CSF, CXCL10, CCL20, XCL-1. In particular adenovirus gene transfer of IL-2, GM-CSF and IL-12 have been extensively studied and results have been promising enough to start clinical trials (Hitt and Gauldie, 2000).

3.2 Aims of the chapter

The aim of this part of the thesis was to construct an adenovector expressing CCL16 and to test CCL16 function as chemoattractant properties of T cells and DC as well as stimulator of CCL2 production by them.

To this aim:

- AdCCL16 was rescued using a site specific recombination method
- The chemoattractant activity of viral produced and recombinant CCL16 was assessed on murine leukocyte subsets
- T cells and immature DC were stimulated with different amount of recombinant CCL16 and the amount of CCL2 released in the supernatants was evaluated.

3.3 Results

3.3.1 Construction of AdCCL16

To study whether delivery of CCL16 in prestablished tumors have antitumor activity an adenovirus encoding CCL16 was generated. AdCCL16 was generated by Cre-mediated homologous recombination in 293 cells (Ng P, 2000).

Two plasmids were used, one contains the entire Ad5 genome, except for key deletions and the Cre-recombinase gene followed by a *lox P* site. The second plasmid, called shuttle, contains an ITR junction, a packaging signal, a promoter, a polyadenilation signal and a *loxP* site.

The cDNA for CCL16 was cloned in the “shuttle” plasmid, PDC316, under the regulation of an exogenous promoter (CMV) and the simian virus (SV40) polyadenilation sequences (see material and methods section 2.2). The other plasmid is PBHGloxDE1,3 Cre that has a complete deletion of the E1 region and a 2653bp deletion in the E3 region of the viral genome. The combination of E1 and E3 deletions results in a cloning capacity of about 8 kb (Ng P, 2000). Other essential features of this Ad genomic plasmid include an ITR junction, necessary for viral replication, a packaging signal, a target sequences for Cre-mediated recombination (*lox P* site) and a cassette for expression of the site specific

recombinase (Cre gene). Cotransfection was performed in the E1 complementing cell line 293 cells as described in materials and methods (section 2.1.2). Recombination between the two plasmids results in excision of the Cre gene and integration of the transgene cassette into the Ad5 genomic plasmid (Fig 3.1). The recombinant Ad vector can replicate in 293 cells and be packed in infectious virions.

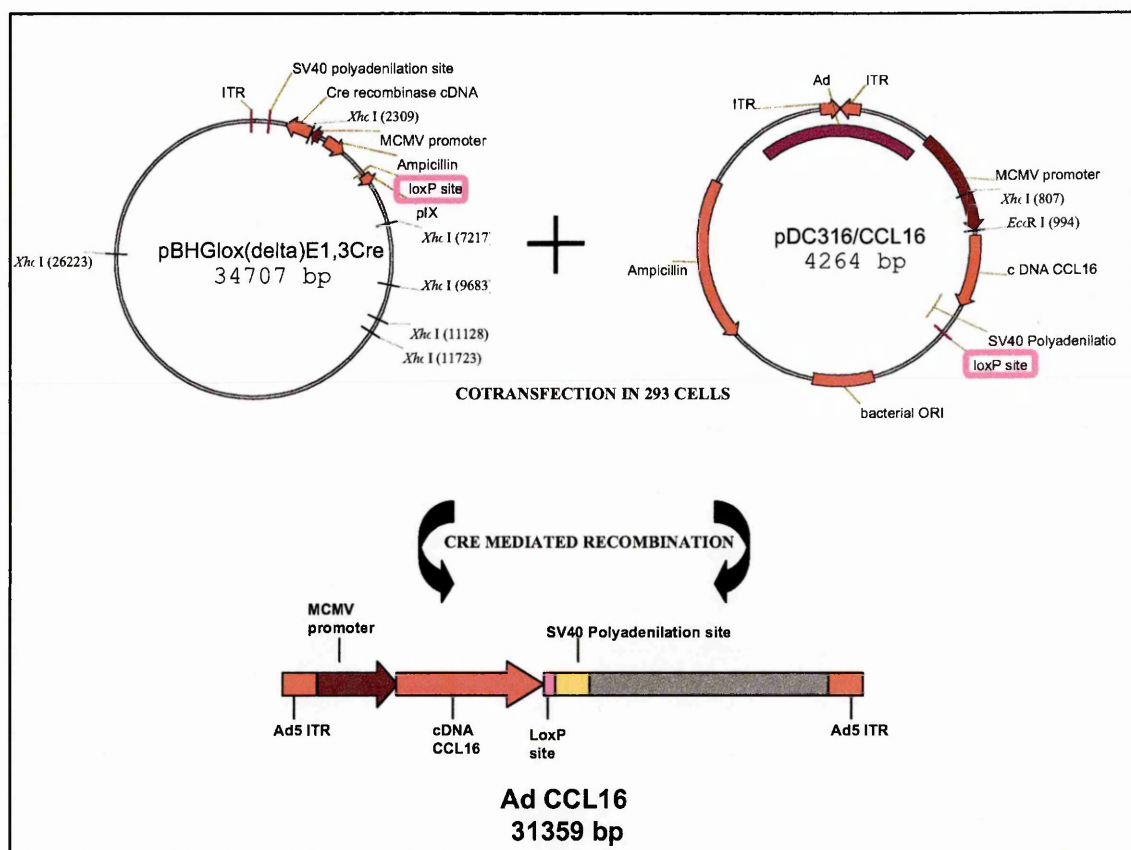


Figure 3.1 Scheme of the method used to generate AdCCL16 adenovector.

Schematic map of the two plasmids that co-transfected into 293 cells recombine to generate AdCCL16. The Cre recombinase mediates the site-specific recombination between the two loxP site (circled in pink).

A number of plaques were obtained and were initially screened by restriction digestion of the DNA from infected 293 cells. Figure 3.2 shows the map of AdCCL16 (panel A) and

the restriction patterns obtained by digestion of four representative plaques (panel B). Subsequent analysis of supernatants from the same plaques by ELISA demonstrated correct expression of the CCL16 protein (Panel C).

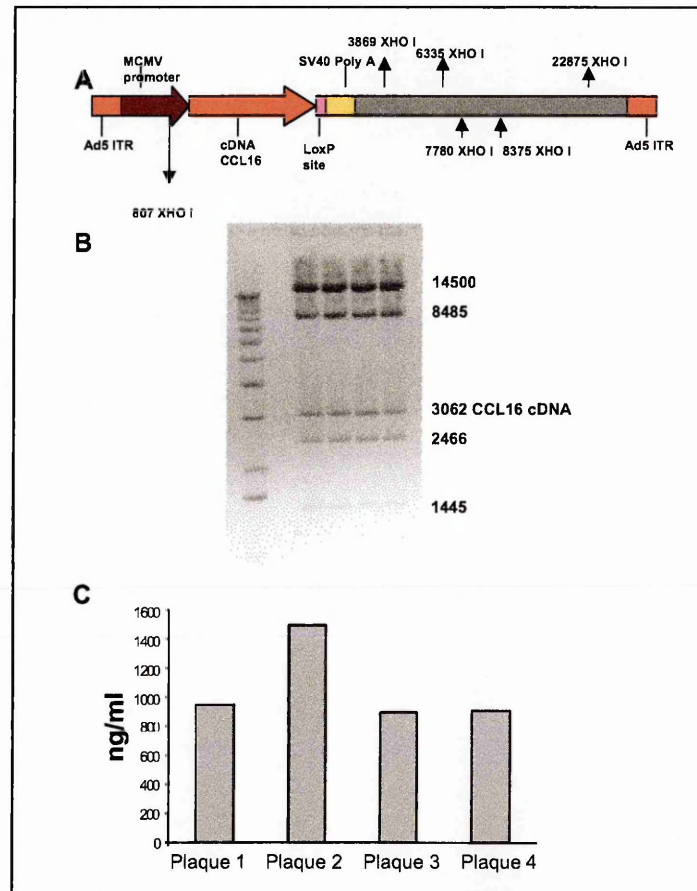


Figure 3.2 Analysis of rescued AdCCL16 viral plaques.

A) Schematic map of AdCCL16 DNA B) restriction enzyme digestion of viral DNA isolated from infected 293 cells, the band containing CCL16 DNA is indicated on the right of the agarose gel. C) CCL16 production by 293 cells infected cells.

As control, an empty adenoviral vector, Add170-3, kindly provided by Dr. Frank Graham (Mc Master University, Hamilton, Canada), was used.

3.3.2 Characterization of chemotactic activity of recombinant and virus produced CCL16

CCL16 was described to be chemotactic for human monocytes and lymphocytes. To evaluate its function on mouse leukocytes, cell migration was assayed by the Boyden chamber methods.

CCL16 either from the supernatant of TSA tumor cells infected with AdCCL16 or the recombinant protein was used. To generate viral produced CCL16, TSA mammary carcinoma cells were infected as described in materials and methods (section 2.3) and the amount of CCL16 in the supernatant was carefully titrated in comparison to known amount of recombinant protein.

Figure 3.3 shows results obtained using splenocytes from BALB/c naïve mice. The viral CCL16 showed a chemotactic activity comparable with the recombinant protein confirming that the protein produced by AdCCL16 is biologically active.

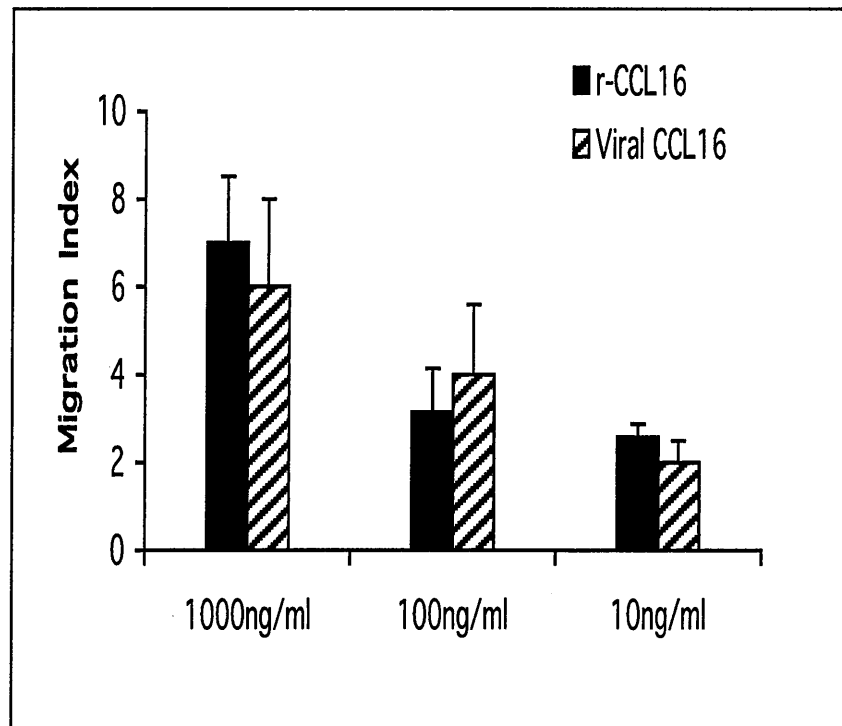


Figure 3.3 Recombinant and virus produced CCL16 is chemotactic for murine splenocytes.

Splenocytes were tested for migration in response to supernatants containing different amounts of recombinant or virus-produced protein. After 3h cells attached to the bottom of the filter, separating the top and bottom of the Boyden chamber, were fixed and counted under a light microscope (40X objective). Assay was performed in triplicate (means of two independent experiments; mean \pm SE). Specific migration (migration index) is expressed as number of cells migrated in the presence of recombinant or viral CCL16/number of cells migrating in medium alone or in supernatant of control virus-infected TSA cells, respectively.

Further analyses were performed on separated leukocytes subsets. For this purpose CD4⁺ and CD8⁺ T cells were purified from total splenocytes using magnetic beads and DC were generated from BM precursors and tested for their ability to migrate in response to both recombinant and viral produced CCL16 (Fig. 3.4 and 3.5).

Both naïve CD4 and CD8 respond to CCL16 in a dose dependent manner moreover the chemotactic response was abrogated by preincubation of cells with pertussin toxin, suggesting that CCL16 mediates its chemotactic activity by G_α-coupled receptors (Fig. 3.4).

As a way to understand the potency of CCL16 in attracting DC, its chemotactic activity was compared to that of CCL20, which is perhaps the best-characterized chemokine known to attract immature DC both in vitro and in vivo (Dieu et al., 1998). As shown in Fig. 3.5 C, CCL16 and CCL20 recruited similar number of DC.

To characterize the ability of CCL16 to attract mature DC, BM derived DC were preincubated with LPS. As shown in Fig. 3.5 D, DC matured with LPS migrated 4-5 times less than immature DC in response to CCL16 and to CCL20 as well.

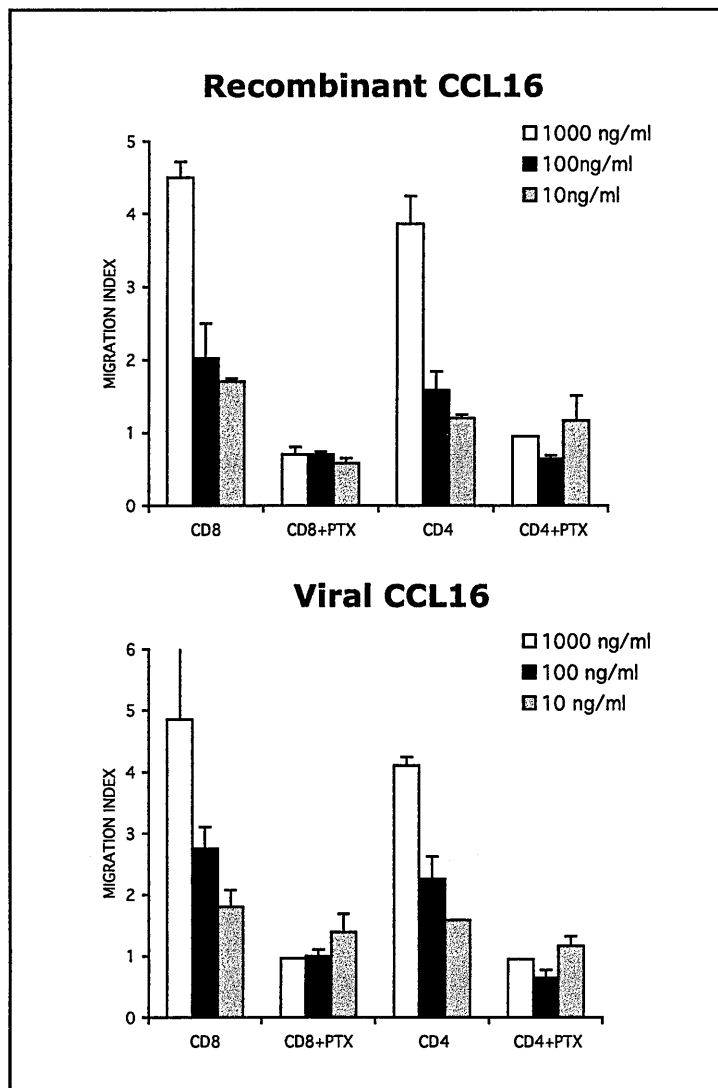


Figure 3.4 CD4, CD8 naïve lymphocytes migrate in response to CCL16.

Purified CD8⁺ and CD4⁺ cells were tested for migration in response to supernatants containing different amounts of recombinant or virus-produced protein. After 3 h for CD4⁺ and CD8⁺ cells attached to the bottom of the filter, were counted. Preincubation with pertussin toxin (PTX) inhibited CCL16-induced migration. Assays were performed in triplicate (means of two independent experiments; mean \pm SE). Specific migration (migration index) is expressed as number of cells migrated in the presence of recombinant or viral CCL16/number of cells migrating in medium alone or in supernatant of Addl70-3-infected TSA cells, respectively.

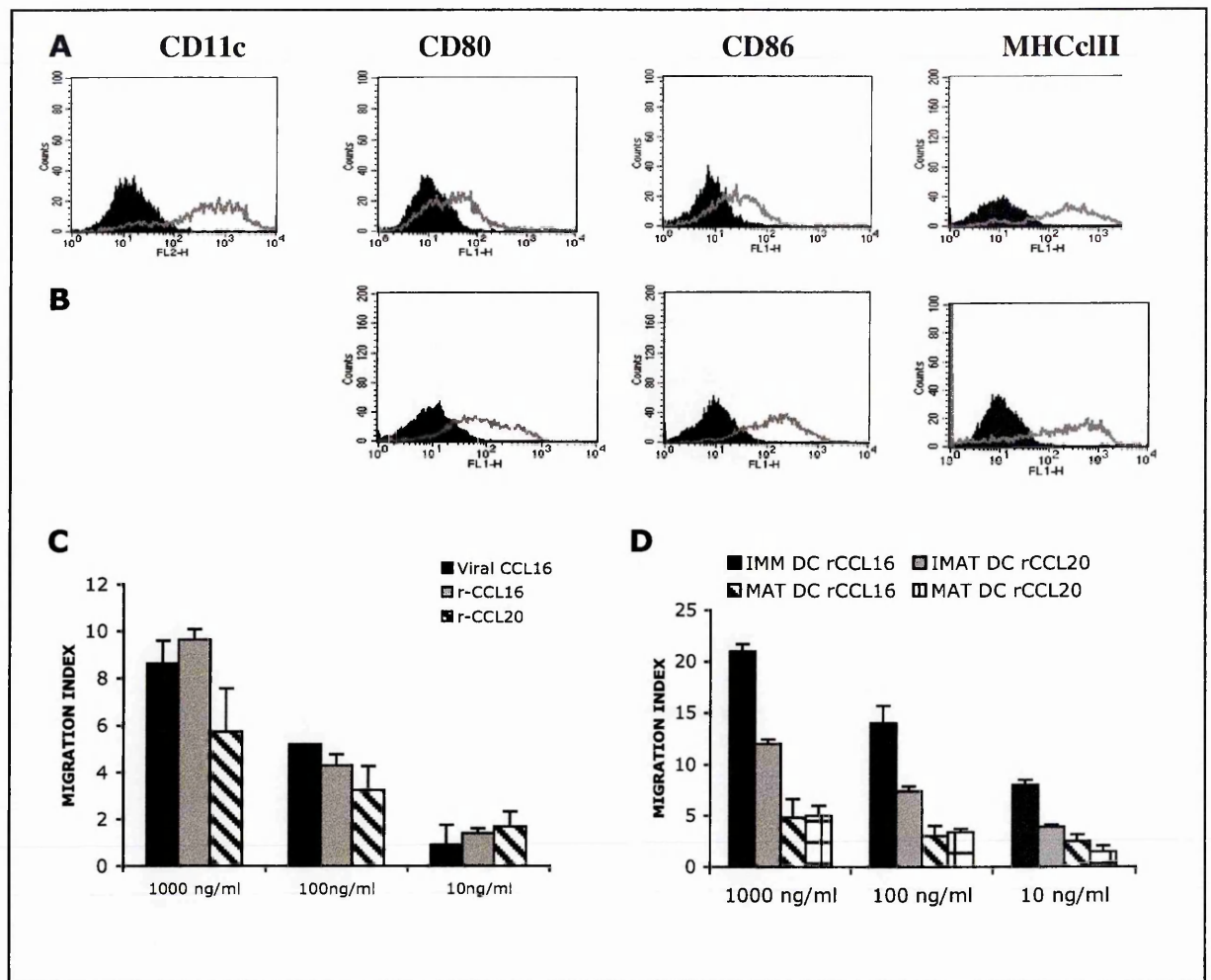


Figure 3.5 DC migrate in response to CCL16.

Phenotypic characterization of immature (A) and mature BM-DC (B). (C) Immature cells were tested for migration in response to supernatants containing different amounts of recombinant hCCL16 or virus-produced hCCL16 or mCCL20. (D) In another sets of experiments immature and mature DC cells were tested for migration in response to recombinant CCL16 and CCL20. Assays were performed in triplicate (means of triplicate; mean \pm SD). Migration index is calculated as above described (Fig 3.4).

3.3.3 CCL16 triggers CCL2 production from T lymphocytes and DC cells

Recombinant hCCL16 has been shown to induce the production of CCL2 in murine macrophages (Cappello et al., 2004). In this section it was evaluated whether CCL16 has similar effects on murine DC and T lymphocytes. DC, CD4 and CD8 T cells were seeded in the presence of different amount of CCL16 and the amount of CCL2 released was assessed 48 h later by ELISA (Fig. 3.6 A-B). Moreover the effect of CCL16 on activated CD4 and CD8 T cells was similarly evaluated (Fig. 3.6 A, right).

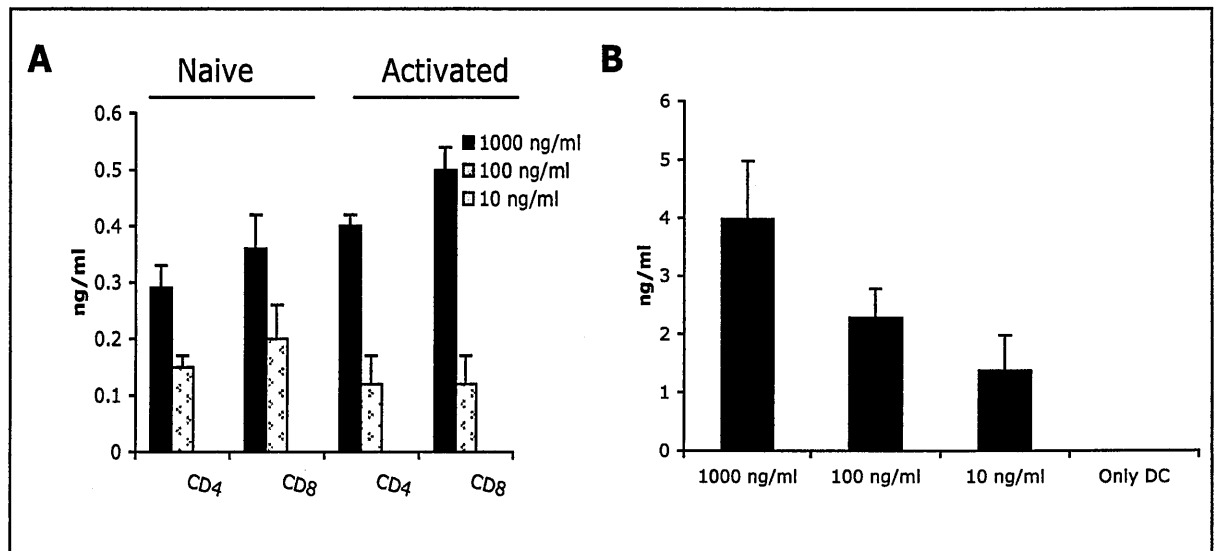


Figure 3.6 CCL16 induces CCL2 expression in DC and T cells.

Purified CD4 and CD8 T cells (A, left) and BM-DC (B) were seeded at a concentration of 1×10^6 /ml in 96 well plates with the indicated amount of rCCL16. To test CCL16 effects on activated T cells, CD4 and CD8 T cells were seeded in 96 well plates pre-coated with anti-CD3 Ab (A, right). Supernatants were collected 48 h later and the amount of CCL2 was evaluated by ELISA. Assays were performed in triplicate (mean \pm SD).

3.4 Discussion

The chapter aim was achieved throughout construction and characterization of an expression vector suitable for efficiently delivery of CCL16 by infection of tumor cells in vivo. Adenovirus-mediated gene delivery was chosen because of its ability to infect tumor cells in vivo, to induce high levels of transgene expression and to provide a certain adjuvant activity in mounting an immune response.

The coding region of human CCL16 was inserted into an adenoviral vector and the chemotactic activity of viral and recombinant protein was confirmed on murine leukocytes. Because CCL16 exists in mice only as a pseudogene the knowledge of its biology is based on human studies (Shoudai et al., 1998; Youn et al., 1998). Youn *et al*, demonstrated that is chemotactic for human monocytes and naïve lymphocytes, without studying whether difference might exist in lymphocytes subsets (Youn et al., 1998). Here it is shown that CCL16 exerts chemotactic activity on murine lymphocytes both CD4 and CD8 T and that the viral produced CCL16 is as active as the recombinant protein (Fig. 3.4). The only previous study performed in mice that might have suggested an effect of human CCL16 in mice was reported by Giovarelli *et al* (Giovarelli et al., 2000), showing that TSA carcinoma cells transfected with the human CCL16 became heavily infiltrated by lymphocytes and DC when injected into syngenic mice.

Both human and murine chemokine receptors are coupled to G_i proteins and cells pre-treatment with pertussin toxin blocks chemotaxis. CCL16 follows this rule also in mice thus suggesting the same receptor might be involved (Fig. 3.4). In human it has been CCL16 is a functional ligand for CCR1, CCR2, CCR5 (Nomiyama et al., 2001) and CCR8 (Howard et al., 2000), all G_i proteins coupled receptors.

Moreover, in this chapter, it is shown that CCL16 exerts chemotactic activity on immature BM-DC to a level comparable to that of CCL20, which is perhaps the best characterized chemokine attracting immature DC. Upon maturation DC loose responsiveness to CCL16

(Fig. 3.5). These results are consistent with reports showing that, upon DC maturation, receptors for inflammatory chemokines as CCR1, CCR2, CCR5, CCR6 are down modulated while, receptor for chemokines expressed in lymphoid organs, CCR7, is upregulated (Sallusto et al., 1998) (Vecchi et al., 1999). A more detailed analysis showed that both human and mouse DC down regulate CCR6 expression upon maturation and become unable to migrate, in vitro, in response to rCCL20 (Dieu et al., 1998).

Capello *et al*, demonstrated that CCL16 enhances the ability of murine peritoneal macrophages to release CCL2 (Cappello et al., 2004). The same cells, stimulated with as little as 100 ng/ml of CCL16, upregulated CD40, CD86 and CD80 costimulatory molecules. Accordingly to these results, it was found that rCCL16 induces release of high amount of CCL2 from mouse DC as well as CD4 and CD8 T cells, with no differences among naïve and activated T cells (Fig. 3.6). Ten times more CCL2 was released by DC with respect to T cells, a value similar to that found in macrophages. However, differently for what reported in macrophages, DC stimulated with CCL16 did not upregulate costimulatory molecules (not shown).

CHAPTER 4: ABILITY OF AdCCL16 TO MODULATE TUMORIGENICITY

4.1 Introduction

In immunotherapy approaches, chemokines are most exploited for their ability to induce migration of dendritic cells (DC) (reviewed in Chapter 1 section 1.3 and (Homey et al., 2002)).

DC recruitment has been described in prestablished B16 melanoma tumors treated with recombinant CCL19, although tumor rejection was incomplete (Kirk et al., 2001a; Kirk et al., 2001b). Other studies also reported DC recruitment to the tumor site, in mice injected intranodally with adenovirus carrying the human CCL20, a gene which , induced rejection in 60-80% of mice in two out of three tumor models tested, and in mice treated with adenovirus encoding CCL22, which was effective in 60% of mice (Guo et al., 2002). However most of investigations have not extensively investigated the actual role of DC in tumor rejection and no compelling animal models for prevention and cure of distant metastases, the critical issue in cancer therapy, has been tested before.

These points were addressed using human CCL16, a chemokine poorly characterized at the functional level. To date only three reports analyzed the potential activity of CCL16 as antitumor agent.

The TSA mouse adenocarcinoma cell line was transfected with human CCL16 cDNA and was shown to be rejected through a massive infiltration of leukocytes (Giovarelli et al., 2000). Rejection of CCL16 was associated with an impressive infiltrate of macrophages, dendritic cells, PMN and T cells and was abrogated by T cells and PMN depletion. This latter data suggests that both innate and adaptive response mediates the rejection of TSA/CCL16 tumors. Mice injected with CCL16-transfected TSA tumor cells were able to reject a contralateral challenge of the parental cells as early as 6 days later, indicating rapid

development of systemic immunity. This latter result suggests that CCL16 accelerate events that underline cross-talk between DC and T cells (Giovarelli et al., 2000).

In another study the antitumor activity of a fusion protein between CCL16 and chTNT-3, a monoclonal antibody previously shown to target tumors by binding to DNA exposed in necrotic zones, was tested on prestablished tumors.

Immunohistochemical studies on CCL16/chTNT-3 treated tumors showed heavy infiltration of CD4⁺ and CD8⁺ T cells, PMNs and CD11c⁺CD11b⁺ myeloid dendritic cells. Despite the abundant leukocytes infiltration only 30-40% of treated tumors showed reduction of tumor growth and only 10% undergo complete regression (Li et al., 2003b).

Authors hypothesized that infiltrated leukocytes might be suppressed by CD4⁺CD25⁺ regulatory T cells. Indeed in another study CCL16/chTNT-3 fusion protein combined with depletion of regulatory T cells results in a significant improvement in tumor rejection rate (70-90%). Number of CD8 T cells or DC at tumor site did not increase following regulatory T cell depletion, suggesting that infiltrate activation status more than its absolute number was affected following T regulatory depletion (Li et al., 2003a).

4.2 Aims of the chapter

The aims of this part of the project were to characterize the antitumor activity of CCL16.

This was done by:

- Infecting in vitro murine tumor cell lines with AdCCL16 and analyzing their growth in vivo;
- Studying the therapeutic effect of AdCCL16 on prestablished tumors;
- Investigating the immunological effector mechanisms responsible for the antitumor effect of AdCCL16 treatment;
- Studying the ability of AdCCL16 treatment to halt metastatic dissemination;

4.3 Results

4.3.1 Infection of murine tumor cells with AdCCL16 and their *in vivo* tumorigenicity

To verify the ability of AdCCL16 to infect murine tumor cells, TSA (H2^d mammary carcinoma) and C26 (H2^d colon carcinoma) cells were infected at different MOI and the amount of CCL16 in the supernatants was assayed by ELISA 24 and 48 h after the infection. 48 after infection C26 released 400 ± 50 ng/ml at 100 MOI and 1200 ± 180 at 200 MOI (Fig 4.1 A). TSA infected cells produced similar amount of CCL16, 530 ± 40 ng/ml at 100 MOI and 1020 ± 200 at 200 MOI (Fig 4.1 B).

Moreover the ability of AdCCL16 to modify the tumorigenicity of C26 and TSA cells was evaluated. For this purpose, cells were infected *in vitro* with 100 and 200 MOI of AdCCL16 or Add170-3 (empty control vector) and after 24 hours, 5×10^4 C26 cells or 1×10^5 TSA cells were injected subcutaneously into BALB/c syngenic animals. There was a significant inhibition of tumor growth of C26-AdCCL16-infected cells compared with cells infected with the control virus although no mice remained tumor free (Fig. 4.2 A-B). Differently, growth of TSA AdCCL16-infected cells was significantly inhibited with 60% of mice free of tumors 50 days after injection. The remaining 40% of the animals showed significant delay in tumor growth and significant prolongation of survival compared to the controls (Fig 4.2 C-D). Only two mouse out of 14 injected with TSA cells infected with control virus at the highest multiplicity of infection remained tumor free (Fig 4.2 C).

Mice that remained tumor free were challenged after sixty days with a second injection of 1×10^5 TSA cells. No mice developed tumors suggesting the presence of immunological memory.

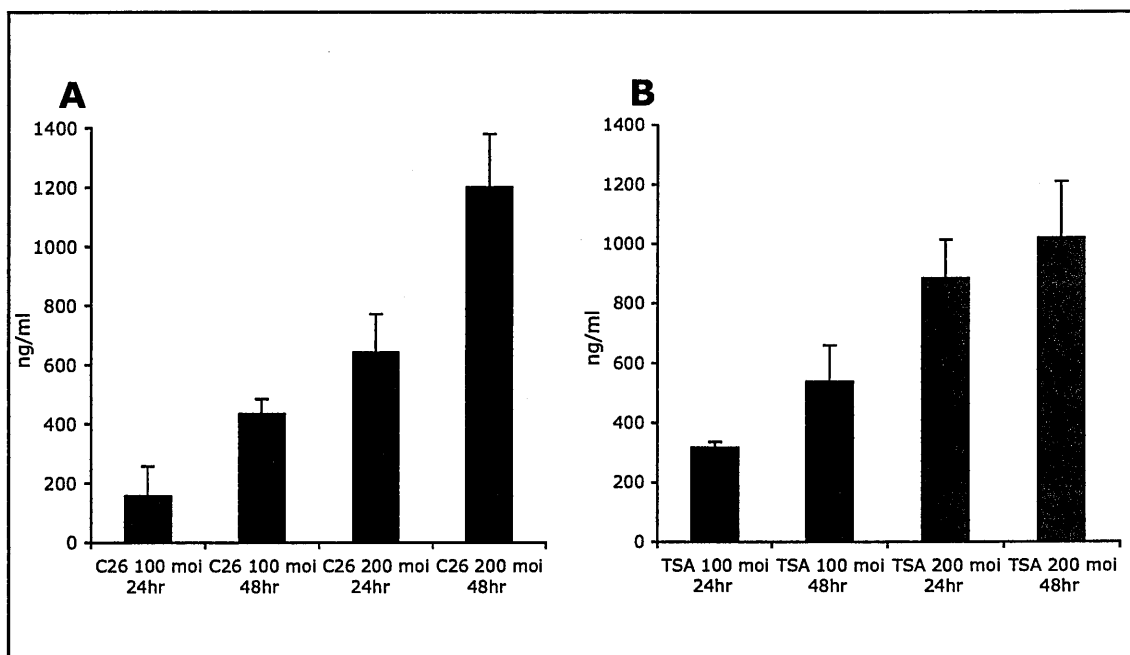


Figure 4.1 CCL16 production by AdCCL16-infected C26 and TSA tumor cells.

5×10^5 cells were seeded in 60 mm cell culture dishes in 5 ml of medium and infected 12 hr later with 100 or 200 MOI. Supernatants, collected 24 and 48 h later, were analyzed for CCL16 secretion by ELISA. Data are given as mean \pm SE of three independent experiments.

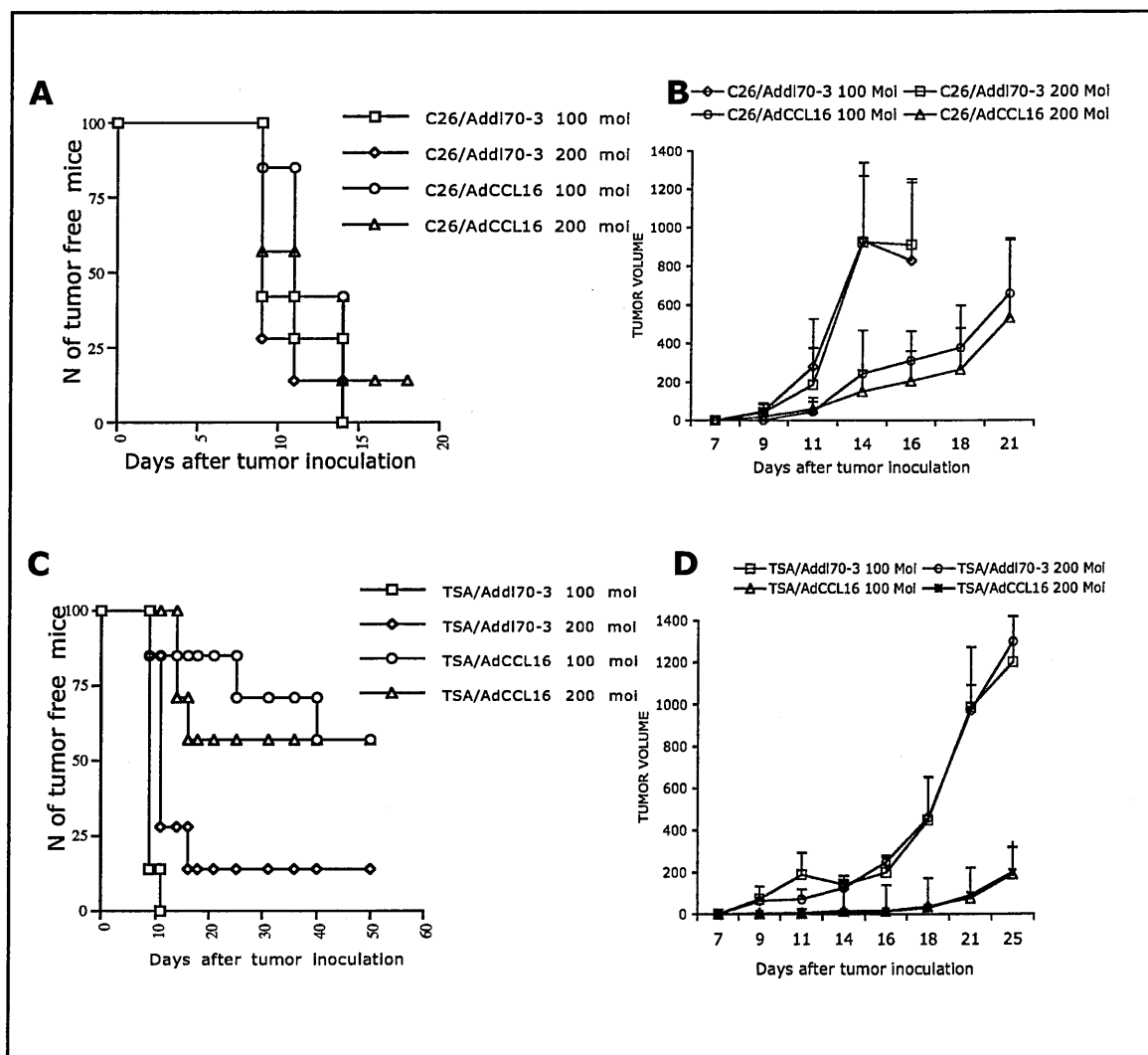


Figure 4.2 Tumorigenicity of AdCCL16-infected C26 and TSA cells.

3x10⁶ C26 or TSA cells were seeded in 100 mm dishes and infected 12 h later with 100 or 200 MOI of AdCCL16 or Addl70-3 empty virus. 24 later cells were collected and 1x10⁵ TSA cells or 5x10⁴ C26 cells were inoculated each in 14 BALB/c mice. Tumor growth is expressed in A and C as number of tumor free mice and in B and D as tumor volumes. In B-D data are given as tumors volumes (mean \pm SD).

4.3.2 AdCCL16 inhibits tumor growth of prestablished tumors

Two tumor models were chosen to investigate the antitumor activity of AdCCL16 on pre-established tumors, TSA and MCA38 (H2K^b colon carcinoma). An unexpected toxicity of both AdCCL16 and the empty Addl70-3 virus inoculated into well-established C26 nodules prevented the use of this tumor model in the studies described in chapter 4 and 5 of the thesis.

At first, the ability of AdCCL16 to infect tumor cells *in vivo* was evaluated. To this aim, TSA and MCA38 nodules were injected with 1×10^9 pfu of AdCCL16 or the empty vector Addl70-3 and collected after 48 h to assay the amount of CCL16 produced *in vivo*. Supernatants from four different nodules were analyzed separately by ELISA. CCL16 production ranged from 120-150 ng/ml for TSA to 70-280 ng/ml for MCA38. No CCL16 production was detected in tumors transduced with the empty vector (Fig. 4.3).

To evaluate the antitumor effect of AdCCL16, mice were injected subcutaneously with 1×10^5 TSA or MCA38 cells, when tumors reached 2-4 mm of diameter (between day 7-11) 1×10^9 pfu of AdCCL16 or Addl70-3 were injected into the tumors. The treatment was repeated four days later. Part of the animals bearing TSA nodules received only the first AdCCL16 treatment as a mean of comparing the effectiveness of one versus two injections. Tumor growth was significantly inhibited especially after two injections, whereas no inhibition was observed in the group treated with Addl70-3 virus and in the untreated group (Fig 4.4 A-B). Fifteen of 49 (31%) mice bearing TSA tumors and 7 of 21 bearing MCA38 tumors (33%) were cured when treated with two injections of AdCCL16. No regressions were observed with control virus. Table 4.1 summarize data from independent experiments.

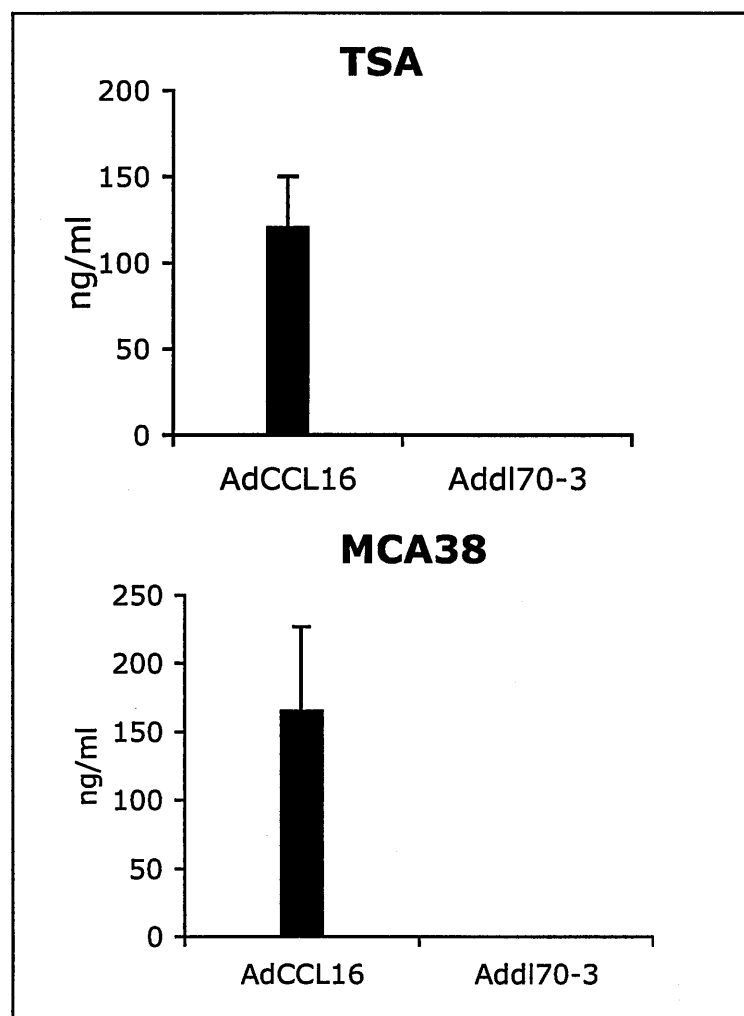


Figure 4.3 Production of CCL16 following intratumoral injection of AdCCL16.

BALB/c and C57/BL6 mice were injected subcutaneously with 1×10^5 TSA cells or MCA38, respectively. When tumors reach 4 mm in diameter they were injected with AdCCL16 or Addl70-3. 48 hr later tumors were collected, treated with collagenase and plated at a density of 1×10^5 cells/ml. 18 hr later supernatants were tested by ELISA for the production of the protein. Four mice were used in each group. Data are given as mean \pm SD.

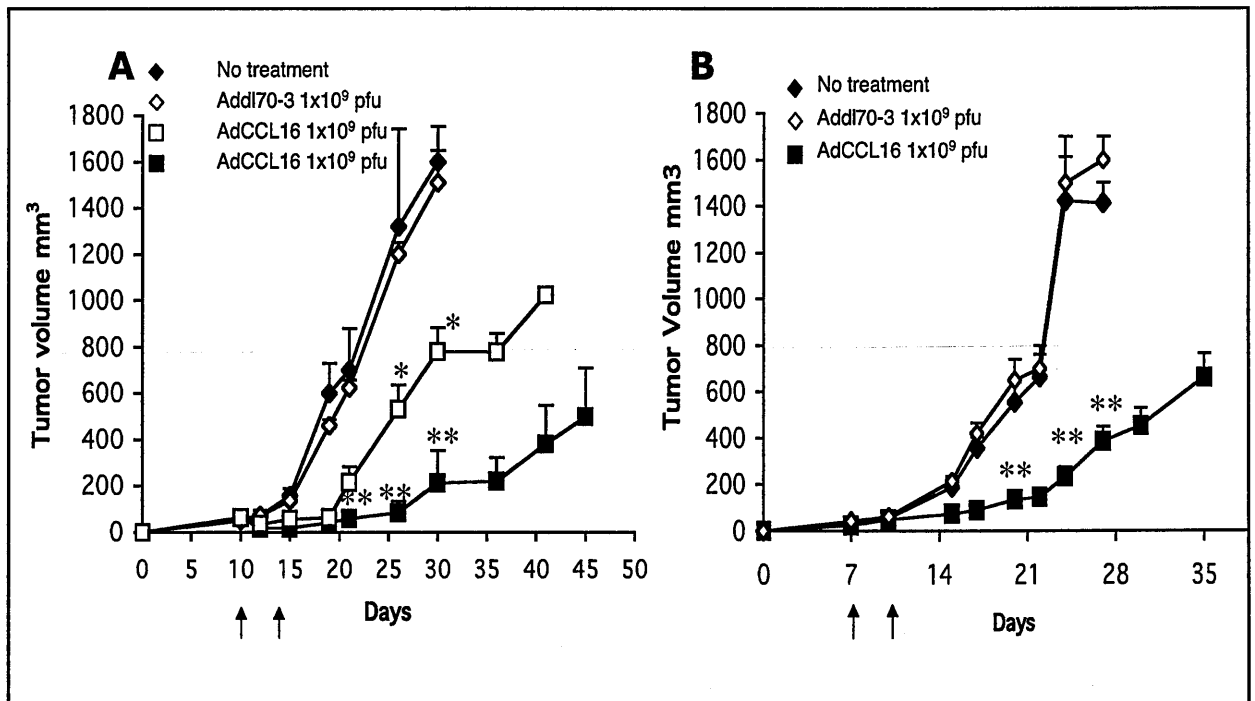


Figure 4.4 Antitumor effects of AdCCL16 on pre-existing tumors.

A) BALB/c mice were injected subcutaneously on day 0 with 1×10^5 TSA cells. When tumors became palpable, mice were left un- treated (◆) or treated with a single (□) or a double injection (■) of 1×10^9 pfu of AdCCL16 or Addl70-3 (◇). B) C57/BL6 mice were injected subcutaneously on day 0 with 1×10^5 MCA38 cells. When tumors became palpable, mice were left un-treated (◇) or treated with a double injection (■) of 1×10^9 pfu of AdCCL16 or Addl70-3 (◆). Data are given as tumors volumes (mean \pm SD). * $P < 0.05$ for (□) versus (◆, ◇). ** $P < 0.01$ for (■) versus (◆, ◇). Seven mice were used in each group. Results are from 1 experiment of 7 for TSA and 3 for MCA38, performed with similar results.

Table 4.1 Response following intratumoral injection of AdCCL16

Treatment ^a	Complete regression %	Rejection following secondary tumor challenge ^b %
<i>TSA tumor</i>		
Addl-70-3	0/49 (0%)	
AdCCL16	15/49 (31%) p<0,001	15/15 (100%)
<i>MCA38 tumor</i>		
Addl-70-3	0/21 (0%)	
AdCCL16	7/21 (33%) p<0,001	7/7 (100%)

a Mice hosting s.c.tumor injected 7-10 earlier were injected intratumor with 10⁹ pfu of AdCCL16 or the control virus at day 0 and 4.

b Mice that have undergone total regression were challenge 90 days later with 2x10⁵ of live cells and monitored for tumor growth.

Data are pooled from independent experiments (7 with TSA and 3 with MCA38).

To evaluate whether mice that rejected TSA and MCA38 tumor following AdCCL16 treatment had developed immunological memory, the ability to reject a secondary challenge with live cells and the presence of memory CTL in their spleens was assessed. All mice rejected the challenge with TSA or MCA38 cells (Table 4.1). For CTL activity, spleens were collected 90 days after primary challenge, restimulated in vitro with irradiated tumor cells or with the specific peptides, corresponding to the known epitopes of the MuLV *env* protein presented in the context of class I MHC L^d (AH1) or class I MHC L^b (KSP). The *env* antigen, corresponding to the envelope protein of an endogenous leukemia virus (MuLV), is expressed by TSA and MCA38 tumor as an immunodominant antigen (Huang et al., 1994a). All mice tested, developed CTL activity although with different intensity among individual animals indicating that the rejection mediated by CCL16 treatment was associated with the development of memory lymphocytes that persisted for up to three months (Fig. 4.5 for TSA tumor model and 4.6. for MCA38 tumor model).

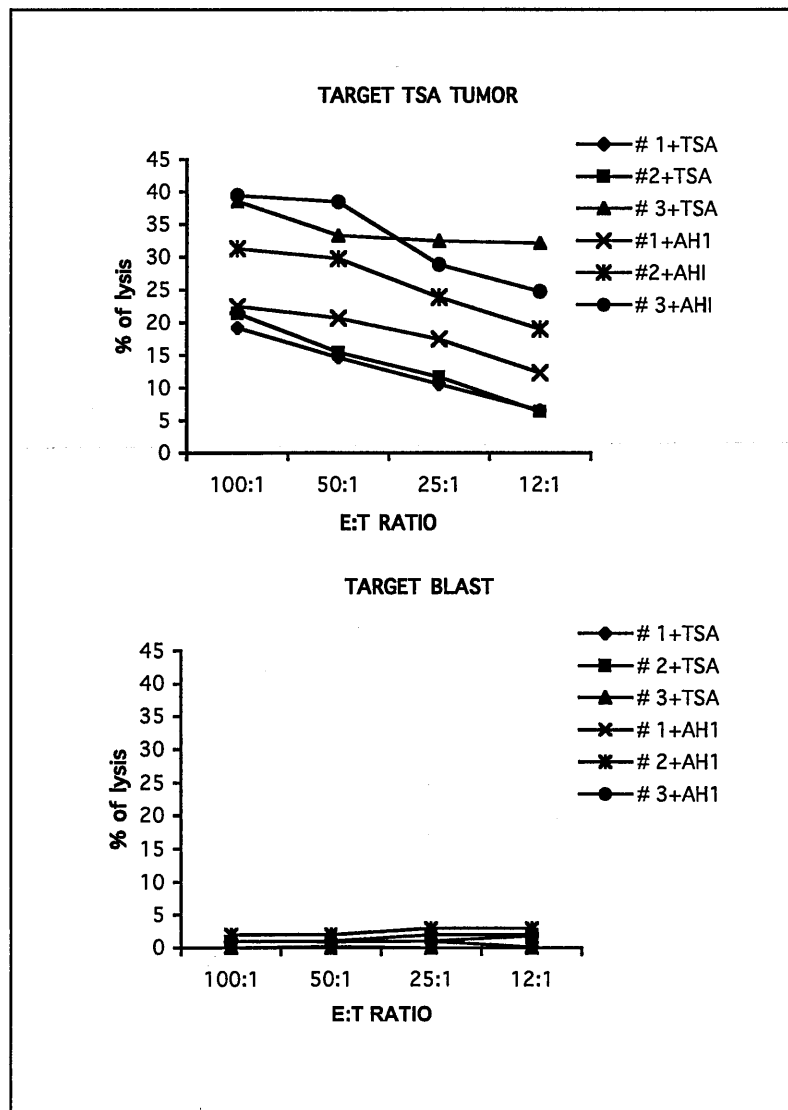


Figure 4.5 Mice that reject TSA tumor following AdCCL16 treatment develop CTL memory response.

Spleen cells from three mice that have rejected TSA tumors were restimulated in vitro with irradiated TSA cells or with AH1 antigenic peptide of TSA. After five days lymphocytes were tested for cytotoxic activity against TSA cells and normal blasts (as negative controls). Data refers to single mice. In vitro stimulus is indicated in the legend. Effector: target ratio (E:T) is indicated.

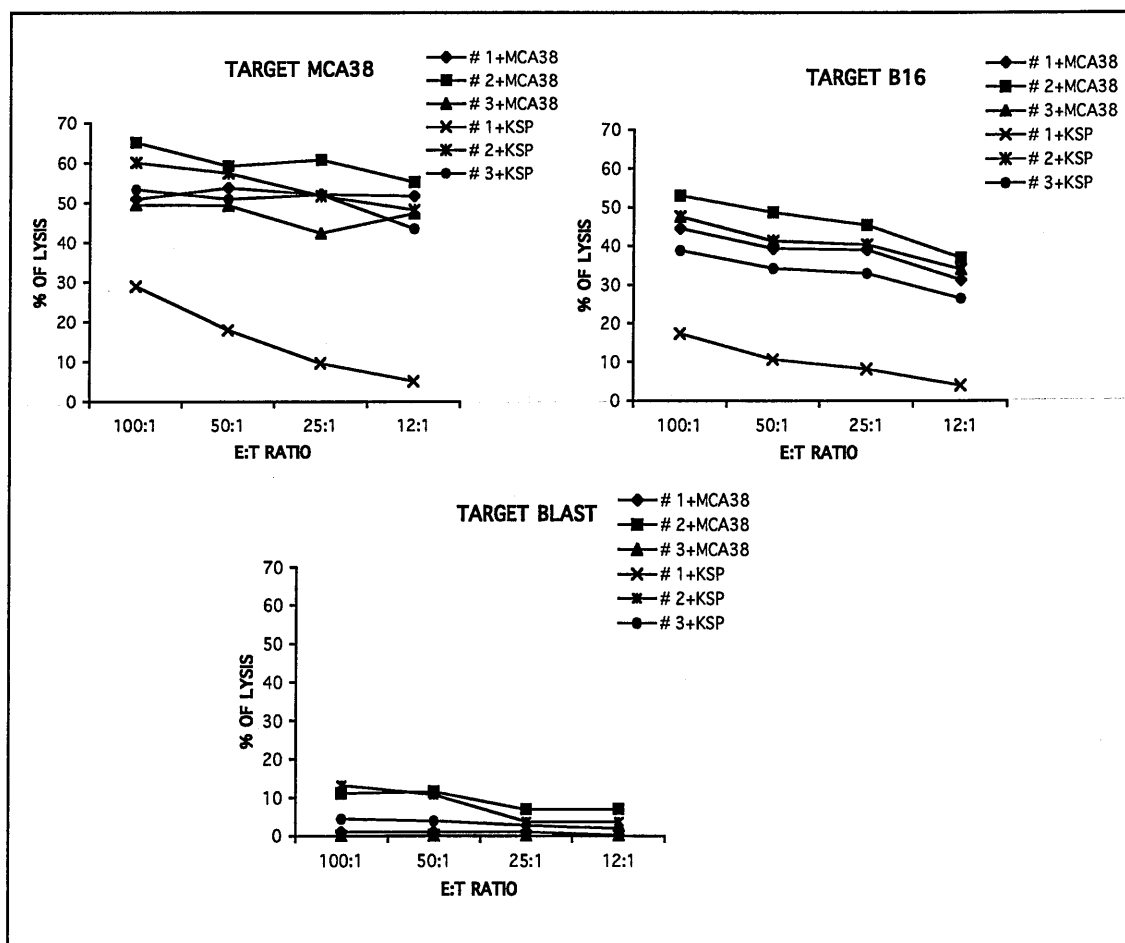


Figure 4.6 Mice that reject MCA38 tumor following AdCCL16 treatment develop CTL memory response.

Spleen cells from three mice that have rejected MCA38 tumor were restimulated in vitro with irradiated MCA38 cells or with KSP antigenic peptide of MCA38. After five days lymphocytes were tested for cytotoxic activity against MCA38 cells, B16 (a melanoma cell lines expressing the *env* antigen) and normal blasts (as negative controls). Data refers to single mice. In vitro stimulus is indicated in the legend. Effector: target ratio (E:T) is indicated.

4.3.3 Morphologic analysis of TSA tumors after AdCCL16 treatment.

Immunohistology has been used to characterize the types of leukocytes infiltrating TSA treated tumors. Tumors were treated at day 0 and 4 with 1×10^9 pfu of AdCCL16 or Addl70-3 and collected for the analysis 1 day after last injection.

AdCCL16 treated tumors showed the presences of reactive cell infiltration inside the tumor mass where small and multiple necrotic foci were also found (Fig. 4.7 A). A marked accumulation of reactive infiltrate with vast necrosis was found on the edge of the tumor and in its surrounding stroma (Fig. 4.7 B). Whereas Addl70-3-injected TSA tumors evidenced much less infiltration and barely detectable necrotic area (Fig. 4.7 C-D).

In-depth analysis of the leukocytes subsets infiltrating treated tumors revealed that although, consistent with the intrinsic immunogenicity of adenoviruses (Yang et al., 1994), in tumors injected with the empty vector leukocyte infiltration was greater than that in PBS-treated tumors, the infiltration induced by AdCCL16 was significantly greater than that of Addl70-3-treated tumors (Table 4.2). All leukocyte subsets examined were more abundant in AdCCL16-treated tumors with a predominance of CD11c⁺, CD4⁺ and especially CD8⁺ T cells (Table 4.2 and Fig. 4.8, A-B). The latter cells were the main source of tumor-associated IFN- γ (Fig. 4.8 C-D), as confirmed by laser scanning confocal microscopy (Fig. 4.8 E-F).

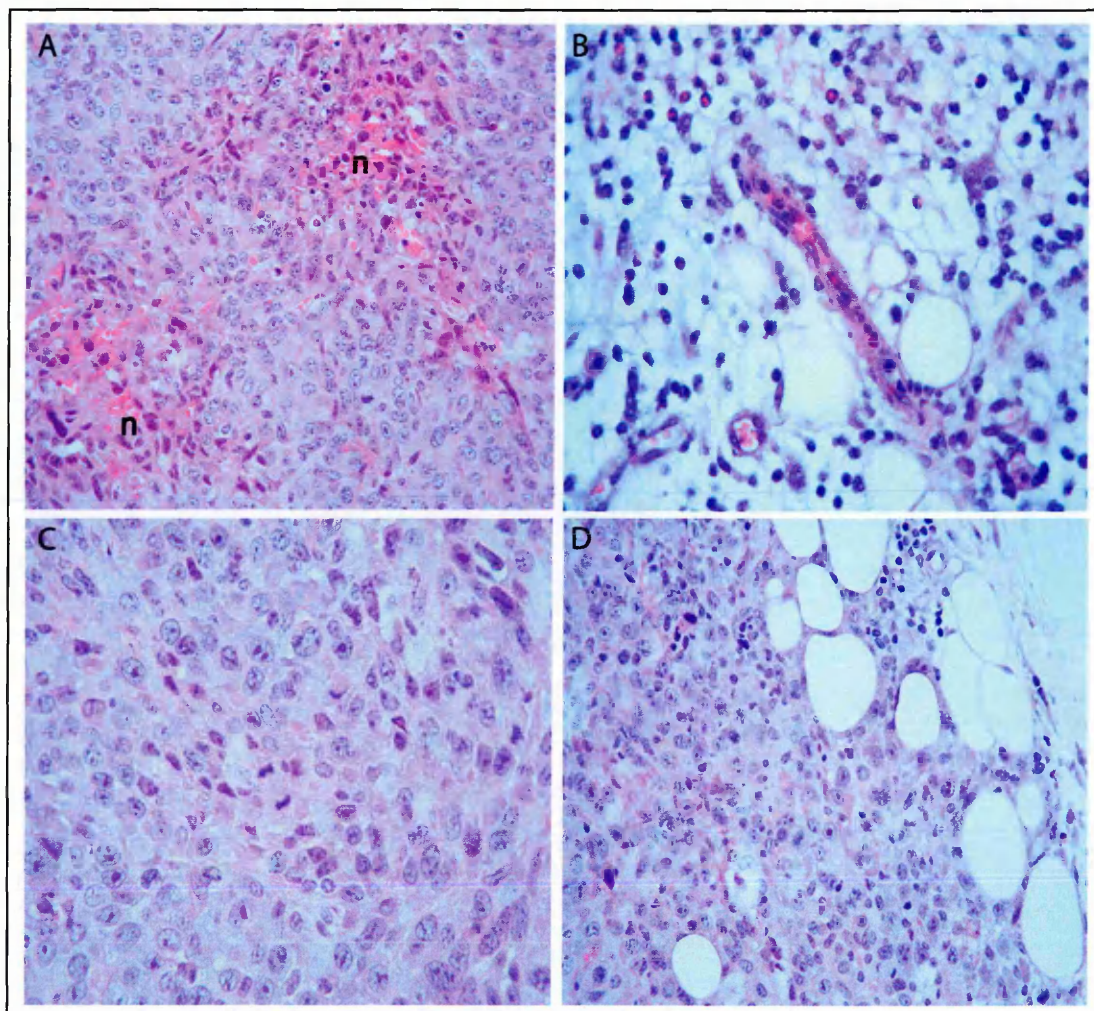


Figure 4.7 Histological analysis of TSA tumors after AdCCL16 or Addl70-3 treatment.

TSA tumors of 3-4 mm were injected at day 0 and day 4 with AdCCL16 or with Addl70-3 and processed for immunoistochemistry analysis the following day. TSA tumors, formed by round to polygonal epithelial cells with frequent mitotic figures, when injected with AdCCL16 evidenced small necrotic foci within the tumor mass (n) (A) and a marked inflammatory reaction particularly in the peripheral areas (B). Addl70-3-injected TSA tumors showed much less inflammatory reaction in both the inner part (C) and in the peripheral area (D) than AdCCL16 treated tumors.

Table 4.2 Reactive cell infiltration induced in TSA tumors after injection with PBS, Addl70-3 or AdCCL16

	PBS			Addl70-3			AdCCL16		
Cells ^a									
Macrophages	21	±	4	28	±	5 ^b	32	±	6 ^b
CD11c ⁺ cells	14	±	3	23	±	3 ^b	32	±	5 ^c
CD8 ⁺ cells	15	±	3	21	±	3 ^b	44	±	9 ^c
CD4 ⁺ cells	12	±	2	24	±	4 ^b	34	±	5 ^c
Granulocytes	3	±	1	7	±	1	8	±	2
NK cells	7	±	2	10	±	2	14	±	3

^a Cell counts performed at x400 in a 0.180-mm² field. At least 3 samples (1 sample/tumor growth area) and 10 randomly chosen fields/sample were evaluated. Results are expressed as mean ± SD of positive cells/field evaluated on cryostat sections by immunohistochemistry.

^b Values significantly different ($p \leq 0.005$) from corresponding values of TSA tumors injected with PBS.

^c Values significantly different ($p \leq 0.005$) from corresponding values of TSA tumor injected with Addl70-3.

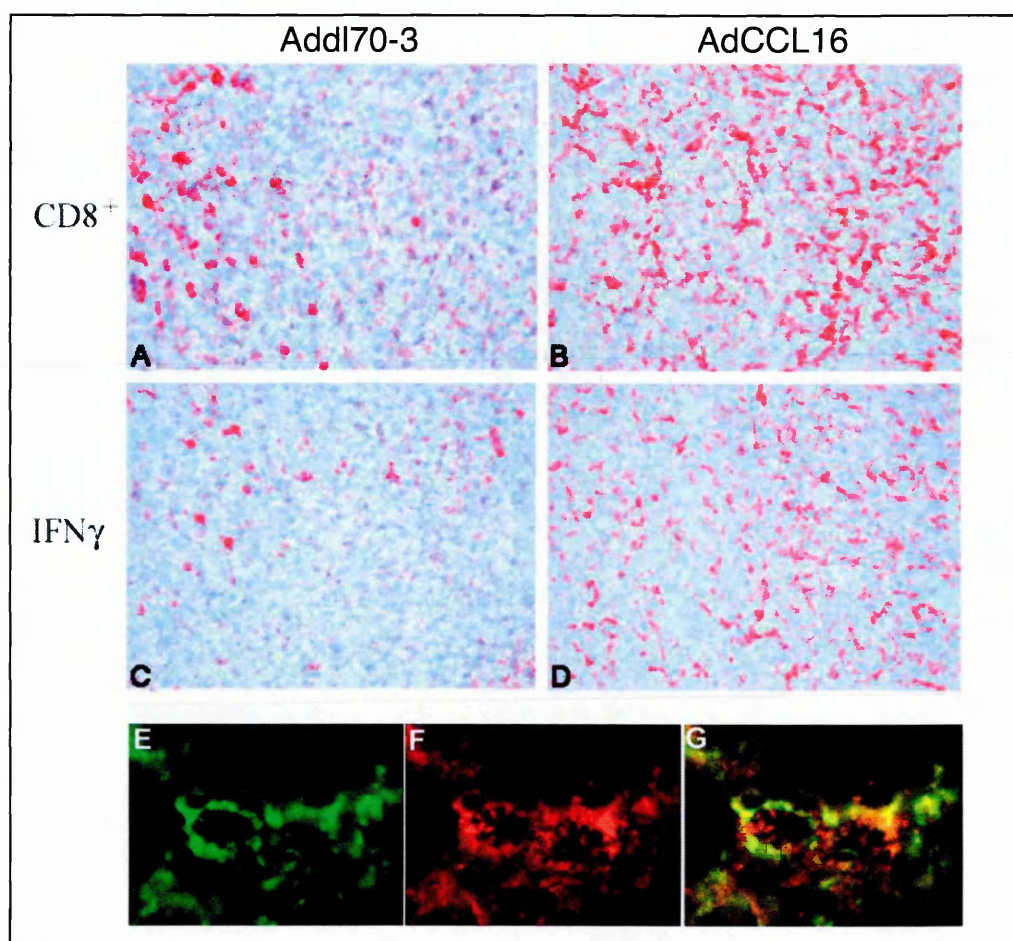


Figure 4.8 CD8⁺ T lymphocytes expressing IFN- γ accumulate in AdCCL16 treated TSA tumors.

TSA tumors of 3-4 mm were injected at day 0 and day 4 with AdCCL16 or with Addl70-3 and processed for immunoistochemistry analysis the following day.

CD8⁺ lymphocytes and cells expressing IFN- γ were among the most represented leukocyte population in AdCCL16 treated tumors (A and B, respectively) than in tumor injected with Addl70-3 (C and D, respectively) (x400). Laser scanning confocal microscopy revealed frequent co-localization of IFN- γ -producing cells (green staining in E) and CD8 cells (red in F), as shown in the yellow regions of the merged image (G) (x 1000)

4.3.4 Kinetics of leukocyte infiltration in AdCCL16 treated tumors

Immunoistochemical analysis indicates that CCL16 promotes accumulation of DC and T cells at tumor site. To characterize the kinetic of leukocyte infiltration, single-cell suspension from TSA and MCA38 tumors were analyzed for the presence of CD4, CD8 and DC at 2, 5 and 8 days after the initiation of AdCCL16 treatment; for comparison tumors treated with Addl70-3 were also analyzed. The number of infiltrating leukocytes was determined by flow cytometry using antibodies for CD4, CD8 and DC cells. The total number of infiltrating cells was normalized to the tumor volume because tumor size varied between and within treatment groups (see materials and methods for details 2.9). In both tumors models, TSA and MCA38, significant influx of CD4, CD8 and DC into tumors was seen 2 and 5 days after AdCCL16 but not control Addl70-3 treatment. Four days after last AdCCL16 treatment, number of infiltrating lymphocytes return to the levels seen in Addl70-3 controls animals (Fig. 4.9).

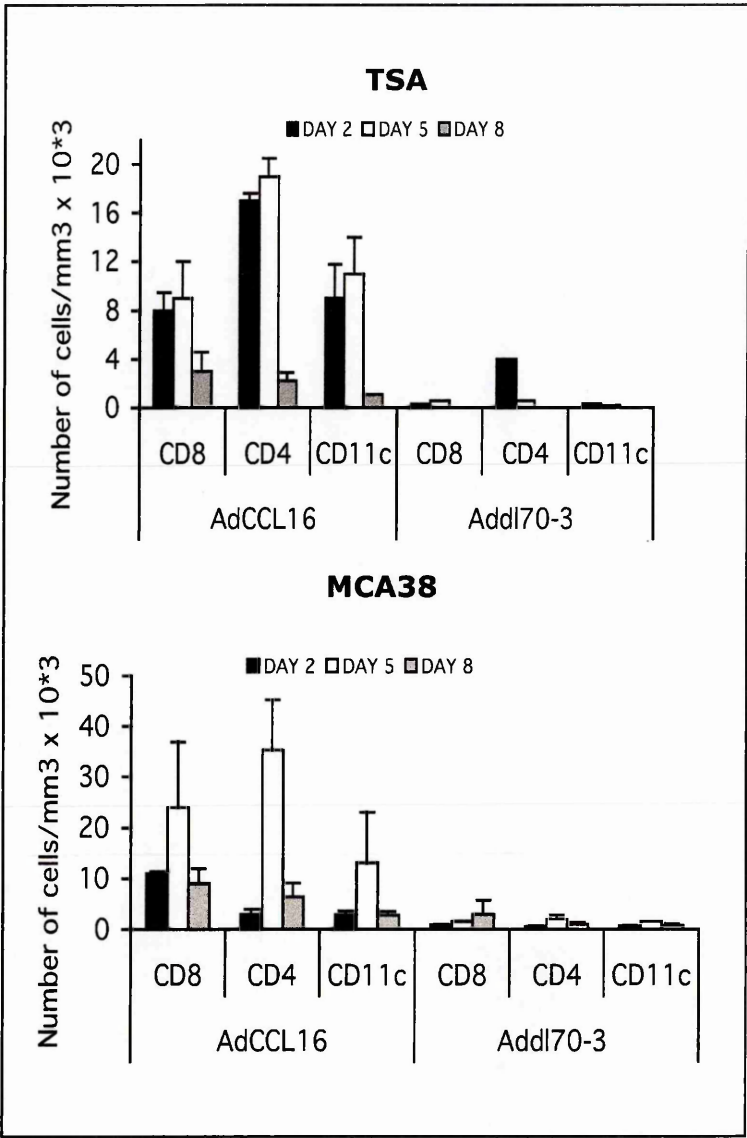


Figure 4.9 Kinetics of leukocytes infiltration following AdCCL16 treatment.

TSA and MCA38 tumors of 3-5 mm were injected at day 0 and day 4 with AdCCL16 or with Addl70-3. Tumors were collected at day 2, 5 and 8 after the first injection and processed according to materials and methods. Number of cells/mm³ were calculated as follows: percentage of positive cells x total number of infiltrating leukocytes x100/tumor volume. 5 tumors mice were analyzed for time point. Data are given as mean \pm SD. Results are representative of 3 independent experiments.

4.3.5 AdCCL16 induces inflammation of draining lymph nodes

One of the most evident consequences of AdCCL16 treatment was the swelling of lymph nodes draining the tumors. To evaluate this phenomenon, inguinal and axillary lymph nodes draining treated tumors were isolated at day 2, 5, 8, 12 and 18 after the first treatment and total number of cell counted. The number of cells was significantly increased in AdCCL16 compared with Add170-3 treated group, in both TSA and MCA38 tumor models (Fig. 4.10). FACS analysis of lymph nodes cells revealed a dramatic increase in the number of CD4⁺, CD8⁺ lymphocytes and CD11c⁺ DC in lymph nodes draining AdCCL16-treated tumors as compared to Add170-3-treated tumors at all points except day 18 when cell count returned to normal. Figures 4.11 and 4.12 shows cell counts of the specific subsets for TSA and MCA38, respectively. No significant differences between Add170-3-treated and untreated mice were observed.

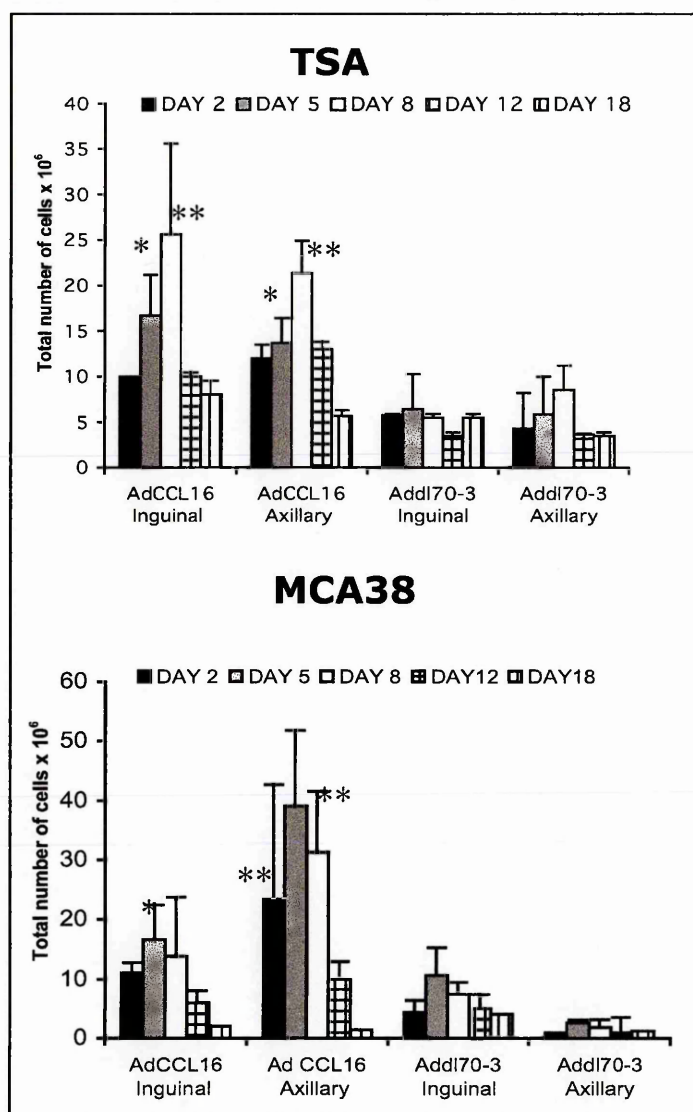


Figure 4.10 Enlargement of draining lymph nodes following AdCCL16 treatment.

TSA or MCA38 tumors of 3-4 mm were injected at day 0 and day 4 with AdCCL16 or with Addl70-3 and inguinal and axillary draining lymph nodes were harvested 2, 5, 8, 12 and 18 days after the first treatment and processed as described in Materials and Methods. Lymph node enlargement was due to increased numbers of cell per lymph node. Three mice were examined at each time point. Data are given as mean \pm SD of number of cells. Results are representative of 2 independent experiments with similar results. Statistical difference is indicated and compares the numbers of cells collected from LN draining AdCCL16 or Addl70-3 injected tumors, * $P < 0.05$; ** $P < 0.01$.

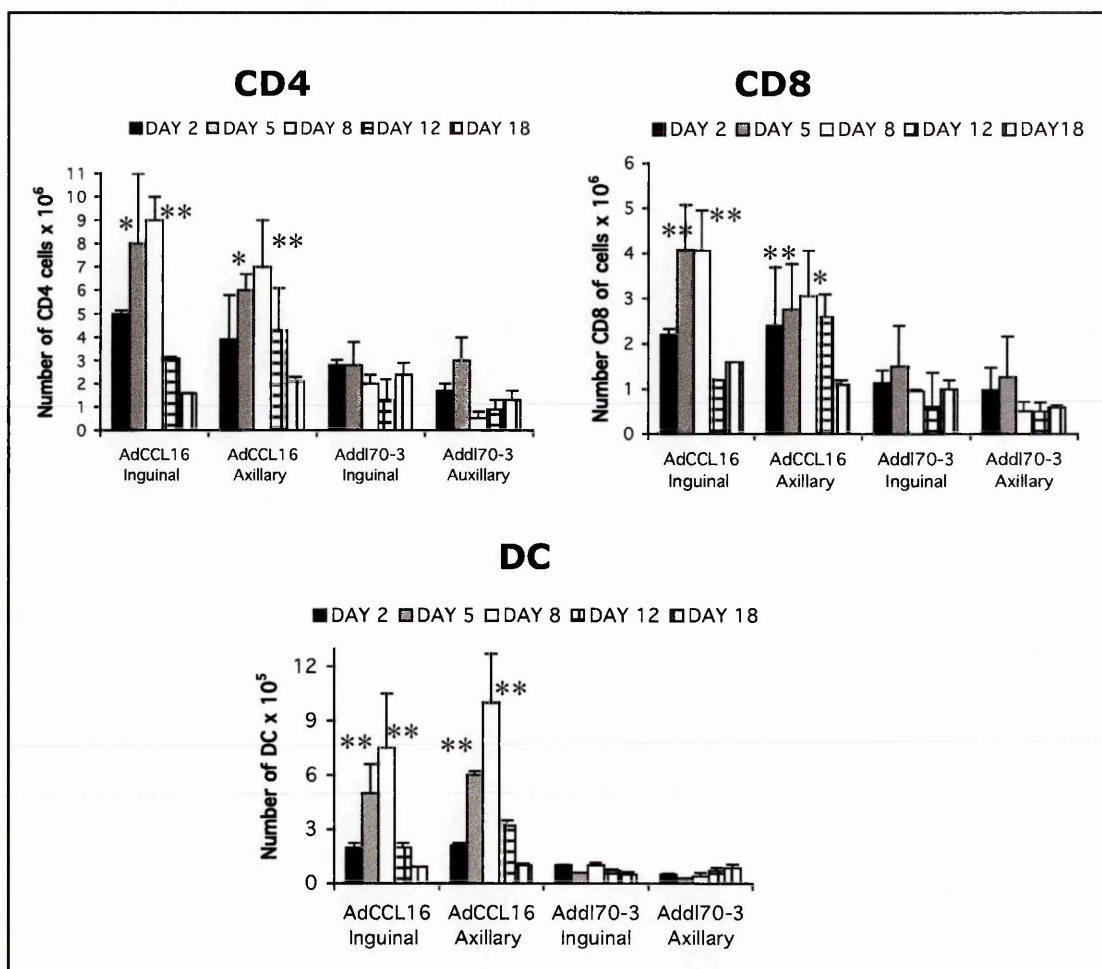


Figure 4.11 Increase number of CD4, CD8 and DC in LN draining TSA treated tumors.

TSA tumors of 3-4 mm were injected at day 0 and day 4 with AdCCL16 or with Addl70-3 and lymph nodes were harvested and analyzed as described in materials and methods. The numbers of CD4⁺ and CD8⁺ lymphocytes as well as of CD11c DC were greatly increased. Three mice were examined at each time point. Data are given as mean \pm SD of number of cells. Results are representative of 2 independent experiments with similar results. Statistical difference is given for each time point and compares the numbers of cells collected from LN draining AdCCL16 or Addl70-3 injected tumors, *P<0.05; ** P<0.01

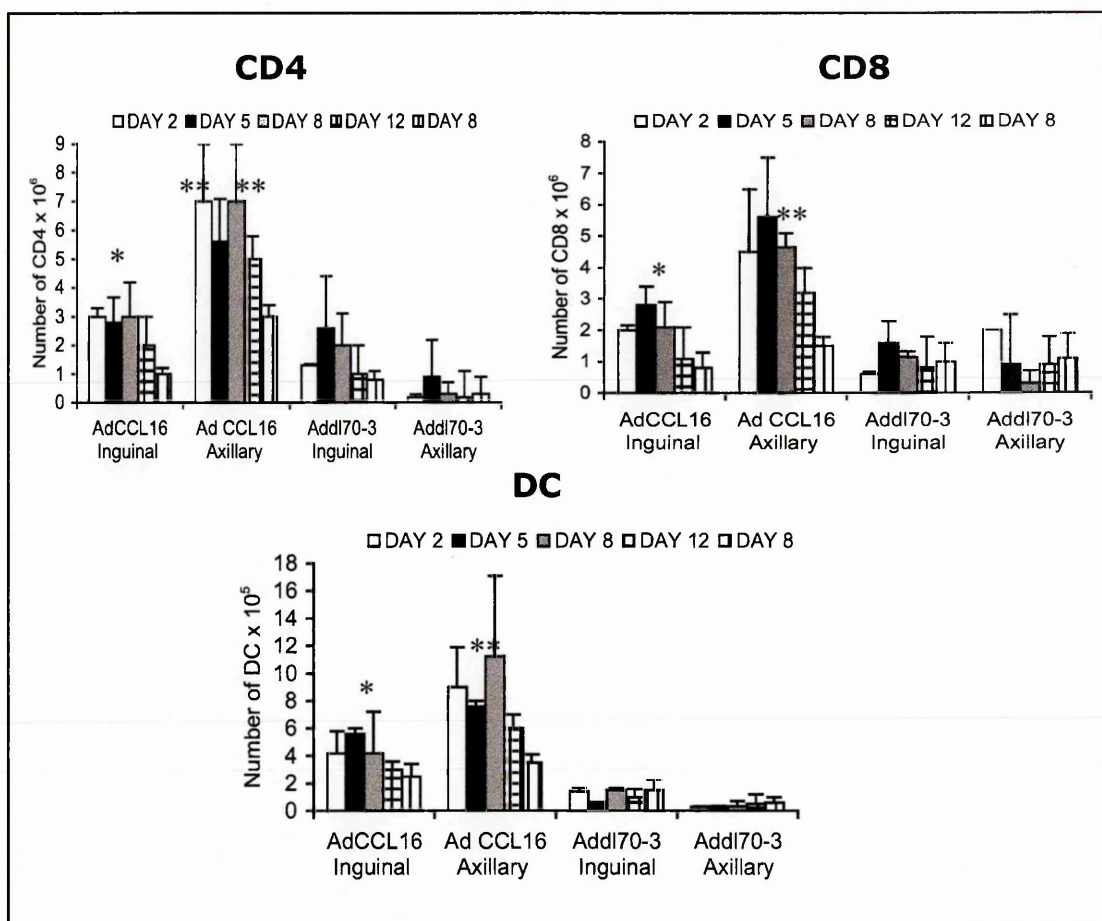


Figure 4.12 Increase number of CD4, CD8 and DC in LN draining MCA38 treated tumors.

MCA38 tumors of 3-4 mm were injected at day 0 and day 4 with AdCCL16 or with Addl70-3 and lymph nodes were harvested and analyzed as described in materials and methods. The numbers of CD4⁺ and CD8⁺ lymphocytes as well as of CD11c DC were greatly increased. Three mice were examined at each time point. Data are given as means \pm SD of number of cells. Results are representative of 2 independent experiments with similar results. Statistical difference is given for each time point and compares the numbers of cells collected from LN draining AdCCL16 or Addl70-3 injected tumors, *P<0.05; **P<0.01

In addition, immunohistochemical analysis of inguinal lymph nodes collected at day 5 showed that CD11c⁺ cells were more represented in the paracortical T cell areas of the lymph nodes draining AdCCL16-injected TSA tumors than in those draining Add170-3-injected tumors (Fig. 4.13 A-B). In the paracortical areas, and sometimes in the medullary cords of lymph nodes draining AdCCL16-injected tumors, IL-12 expression was detected in cells with DC morphology (Fig. 4.13 C-D).

To test the loco-regional activation of T lymphocytes from the draining lymph nodes, IFN- γ was measured by ELISA of total lymphocytes while the percentage of IFN- γ producing cells was evaluated by FACS analysis of CD4⁺ and CD8⁺ subsets. To this extent lymph node cells collected 5 and 8 days after first treatment were restimulated in vitro with plate-bound anti-CD3 Ab and IFN- γ was measured by both ELISA and FACS analysis. Cells from AdCCL16-treated mice produced significantly higher amounts of IFN- γ than cells from mice treated with control virus (Fig. 4.14 A) and the majority of both CD4⁺ and CD8⁺ cells actively produced the cytokine (Fig. 4.14 B).

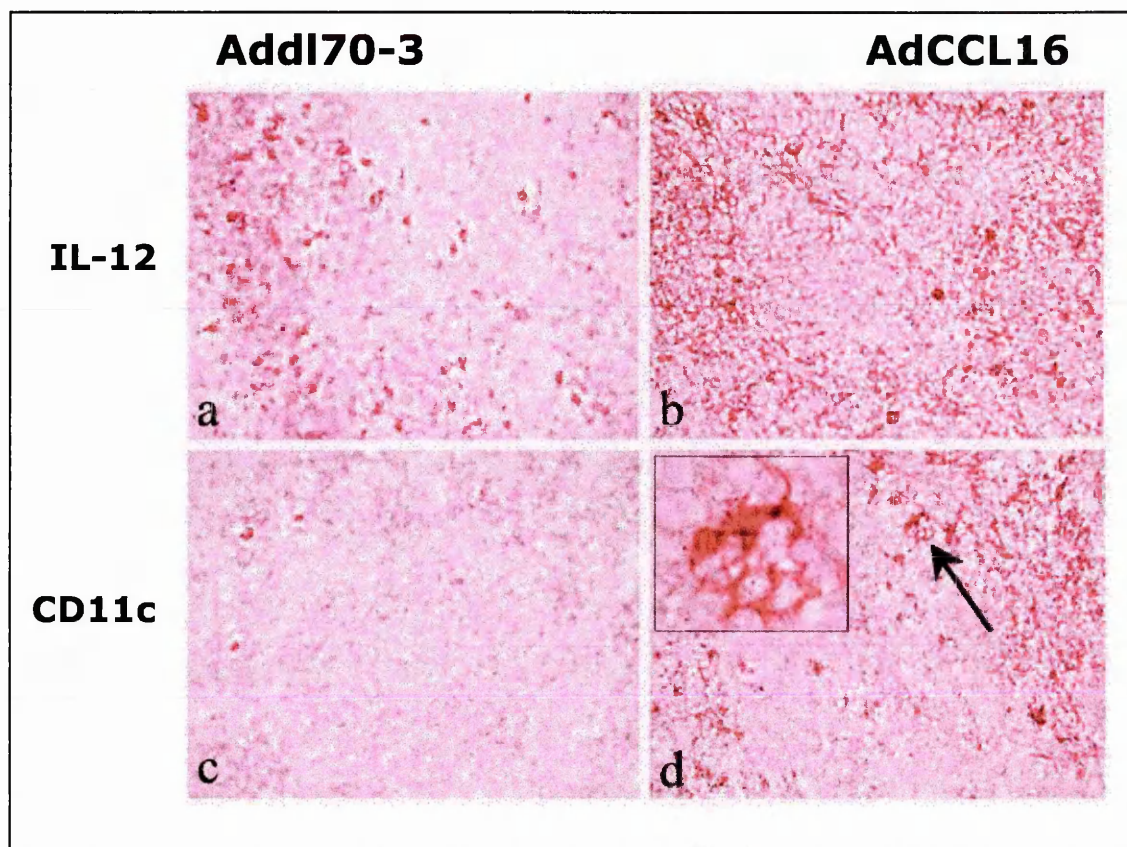


Figure 4.13 Immunohistochemical analysis of inguinal lymph nodes draining TSA tumors on day 5 after treatments.

CD11c+ cells were more represented in the paracortical T cell areas of the lymph node draining AdCCL16-injected tumors (*B*) as composed with those draining Addl70-3-injected tumors (*A*). In addition, lymph node draining AdCCL16-injected TSA tumors revealed distinct expression of IL-12 in cells with cytoplasmatic projections identifiable as DC (the insert shows the detail indicated by the arrow at x1000 magnification) inside the paracortical areas and sometimes in the medullary cords (*D*). By contrast, the IL-12 expression was barely detected in the lymph node draining Addl70-3-injected tumor (*C*) (x400).

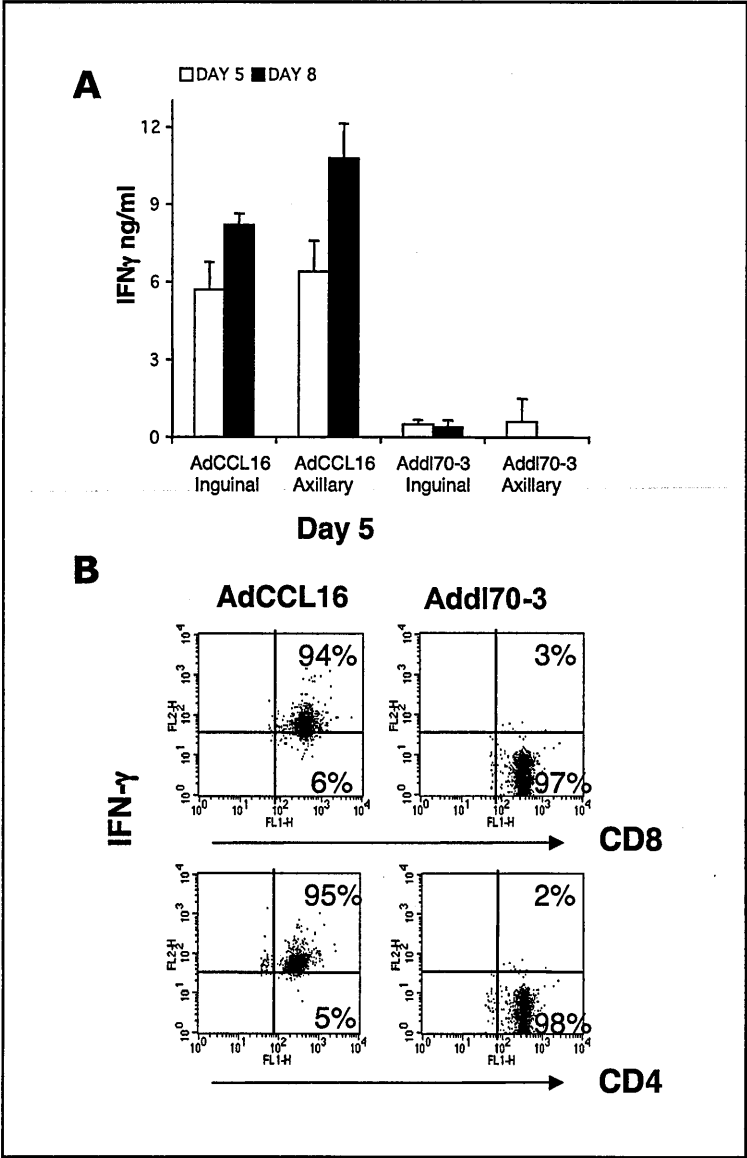


Figure 4.14 Production of IFN- γ by lymph nodes draining treated tumors.

TSA tumors were injected at day 0 and day 4 with AdCCL16 or with Addl70-3 and lymph node cells were isolated on day 5 and 8 after the first adenoviral injection. IFN- γ production was evaluated by ELISA (A) and by mouse IFN- γ secretion assay (B) as described in Materials and Methods. Three mice were analyzed for each time point. Data in (A) are given as mean \pm SD. IFN- γ level ($p < 0.05$). Flow cytometry plots in B show the expression of IFN- γ among gated CD4 $^{+}$ or CD8 $^{+}$ cells; the percentage of positive cells is indicated in the top right corner of the plot. Results are representative of 2 independent experiments.

4.3.6 DC in lymph nodes draining AdCCL16-treated tumors are loaded with TAA and stimulate TAA- specific T lymphocytes

Since DC proliferation in lymph nodes is unlikely (Flores-Romo, 2001), their accumulation in these nodes following AdCCL16 treatment suggests their migration from the tumor. To address this point, CD11c⁺ DC from lymph nodes draining AdCCL16-treated tumors were purified using magnetic beads and used to stimulate IFN- γ production by the T cell clone E/88, as an indication of TAA capture by DC. The CD8 T cells clone, E/88, specifically recognizes the AH1 epitope (Rodolfo et al., 1991) (scheme of the experiment is given below in Fig. 4.15).

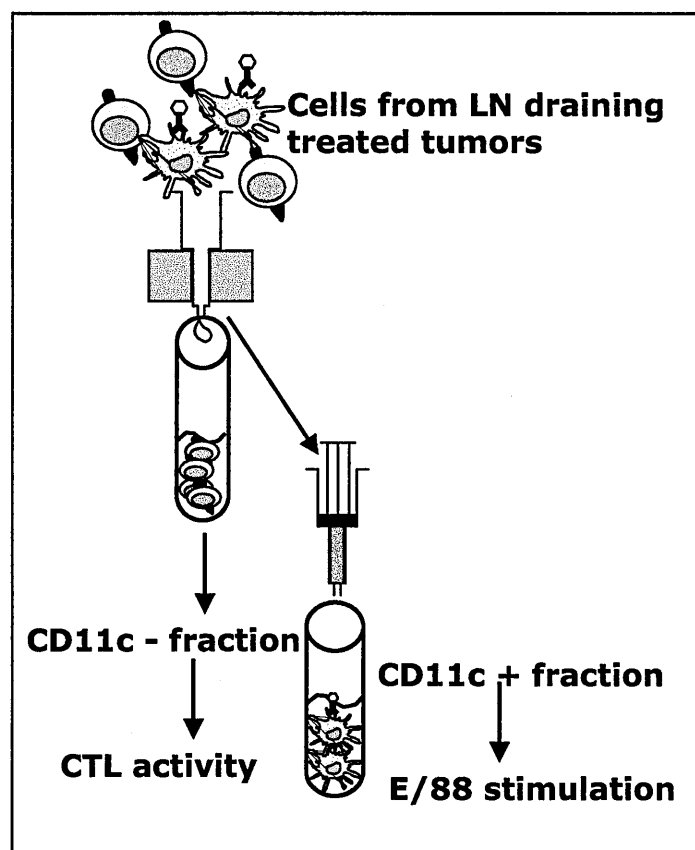


Figure 4.15 Scheme of the experiment presented in Fig. 4.16

As shown in Fig. 4.16 A, E/88 cells produced IFN- γ when cocultured with DC from lymph nodes of treated mice, whereas no IFN- γ production was observed by DC alone, E88 alone or by the CD11c⁻ fraction of the magnetic DC separation containing lymphoid cells. TSA tumor cells were used as positive controls. The low yield of DC from lymph nodes draining tumors treated with Addl70-3 precluded their use as controls in this assay.

The CD11c⁻ fraction of the magnetic separation, containing lymphoid cells, was restimulated for five days in the presence of irradiated tumor cells or the specific AH1 peptide and then used to test CTL activity. Lymphocytes from the CD11c⁻ fraction from AdCCL16 but not Addl70-3-treated tumors elicited specific CTLs against TSA cells, AH1-pulsed blasts cells and C26 colon carcinoma cells which share the same TAA with TSA (Fig. 4.16 B).

Together, these results suggest that DC infiltrating the lymph nodes took up and processed the TAA for presentation to CTL clones as well as for priming of naïve T cells in the draining lymph nodes.

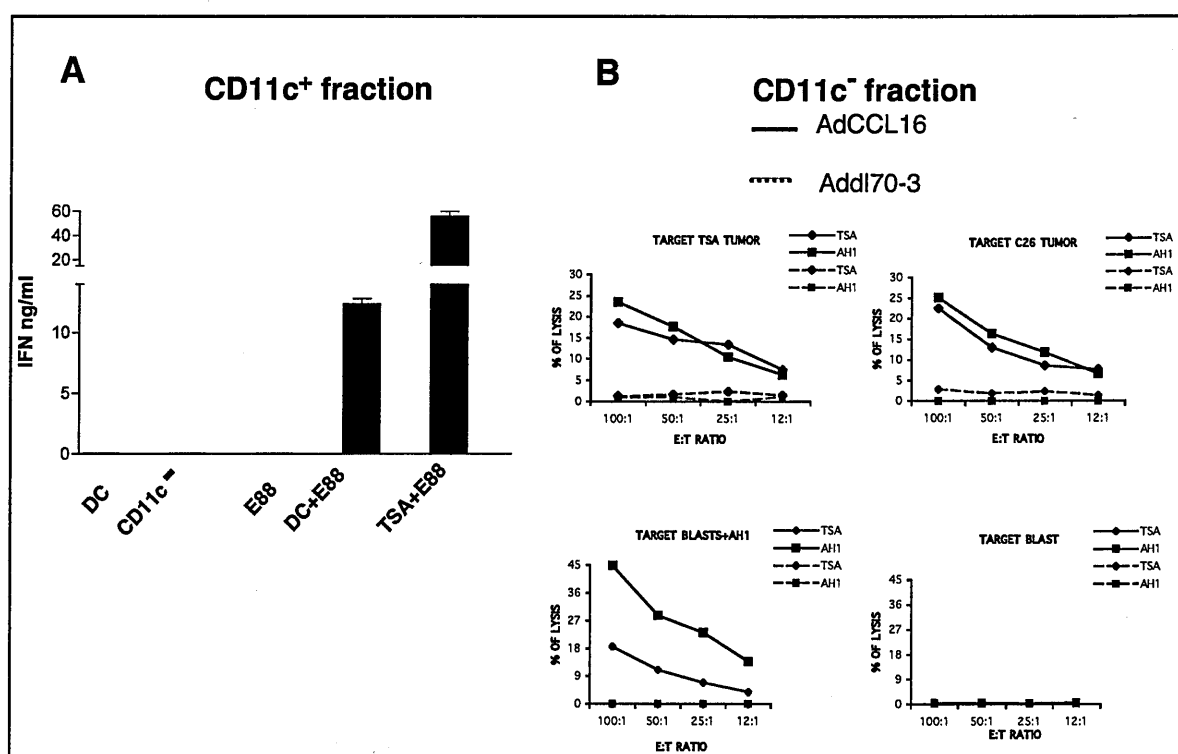


Figure 4.16 Functional characterization of DC from lymph nodes draining treated tumors.

Tumors were treated with AdCCL16 or Addl70-3 at day 10 and 14 from challenge. Two days after the last treatment, draining lymph nodes were isolated and DC were separated by magnetic beads. Two lymph nodes pools, each from three mice, were processed independently. (A) E88 (1×10^5) cells were incubated with stimulating cells at a ratio of 1:1 for TSA cells and 1:2 for purified DC for 18 h. DC, E88 or CD11c negative fractions were incubated alone as negative controls. IFN- γ in the medium was tested by ELISA (mean \pm SD relative to the two independent groups). (B) Lymphocytes from the CD11c negative fraction were restimulated in vitro with TSA cells or AH1 peptide and tested 5 days later for CTL activity. Results are representative of 2 independent experiments.

4.3.7 Antitumor effects is abrogated in mice lacking T cells or in IFN γ -KO mice

To evaluate the role of specific effector cells in AdCCL16 antitumor activity CD4⁺ or CD8⁺ cells were depleted in tumor bearing animals by means of specific antibodies. In both CD4 and CD8 depleted animals the effect of AdCCL16 was abrogated (Fig. 4.17, A-B). The increase production of IFN- γ in LN of mice treated with AdCCL16 (Fig. 4.14) suggests that this cytokine may play a key role in the antitumor activity mediated by CCL16. To test this hypothesis IFN- γ KO (GKO) mice implanted with TSA tumors were treated with AdCCL16 or control virus. In these mice AdCCL16 treatment of tumors was no longer inhibitory (Fig. 4.17 C).

These results indicate that AdCCL16 antitumor activity depends on the presence of both CD8⁺ and CD4⁺ T cells and involves IFN- γ .

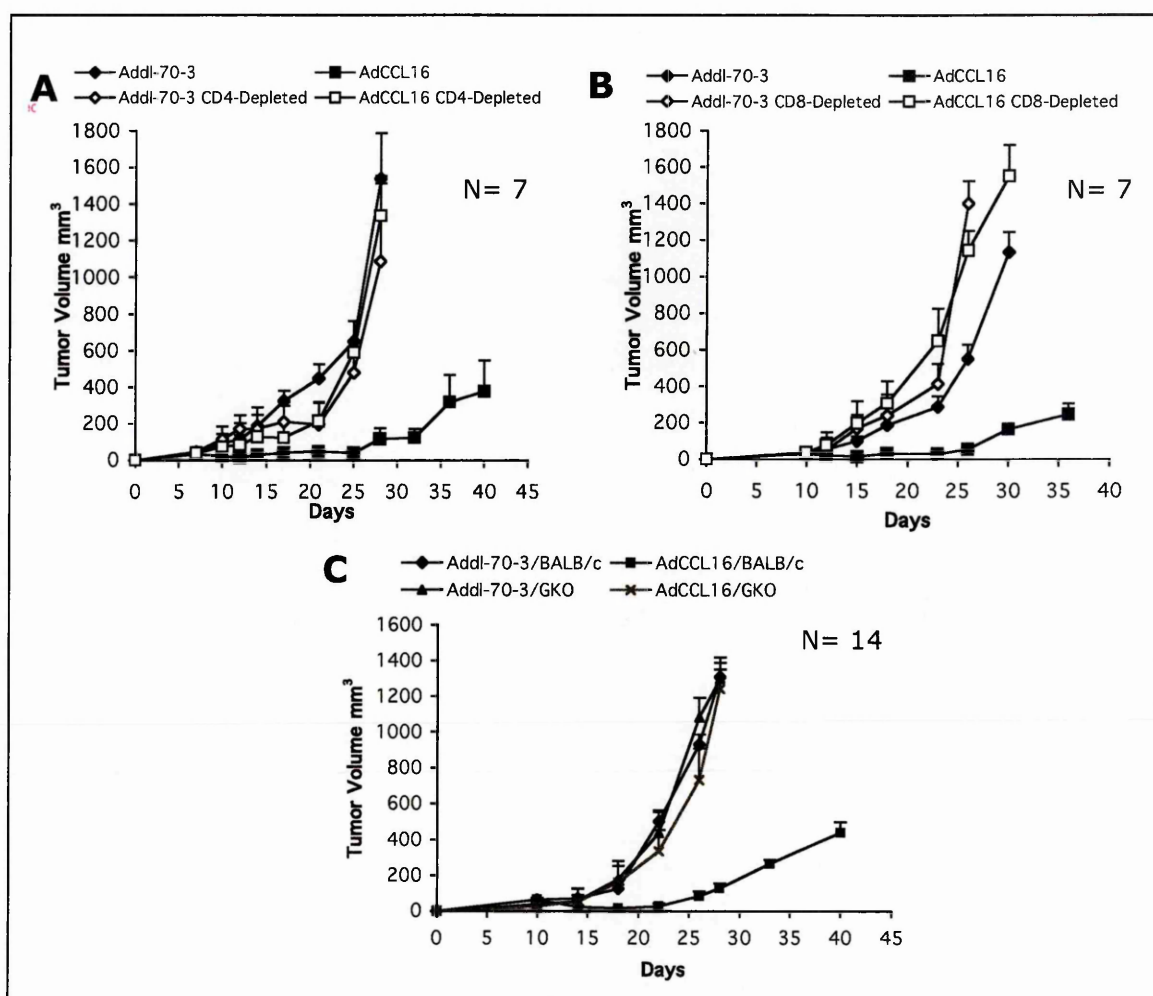


Figure 4.17 Contribution of CD4⁺, CD8⁺ cells and IFN- γ to AdCCL16 anti-tumor effect.

Tumor growth was monitored in mice depleted of CD4⁺ cells (A), CD8⁺ cells, (B) and IFN- γ -knout-out mice (GKO) (C) treated with AdCCL16 or Addl70-3. Number of mice is indicated in each panel. Data are given as mean \pm SD of tumor volumes.

4.3.8 AdCCL16 treatment of metastatic disease

Despite the strong pro-inflammatory activity and the initiation of an effective immune response, intralesional administration of AdCCL16 lead to tumor rejection in only 31% of mice. While addition of costimuli and/or cytokines might have increased the cure rate, it was preferred to focus on the potential usefulness of AdCCL16 in preventing metastatic spread. Perhaps the most compelling model for studying spontaneous metastases, at least on a BALB/c background, is the 4T1 mammary carcinoma (Pulaski and Ostrand-Rosenberg, 1998), since metastases spread immediately after tumor injection and the process is not impaired by removal of primary tumor (Pulaski et al., 2000).

As in TSA model, treatment of the 4T1 established tumors with AdCCL16 significantly inhibited primary tumor growth, but tumors eventually regrew and no complete rejection was observed (Fig. 4.18).

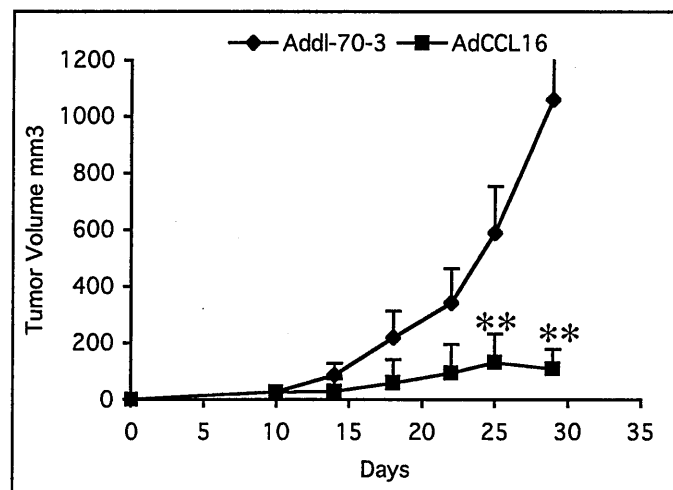


Figure 4.18 Antitumor effects of AdCCL16 on 4T1 pre-existing tumors.

BALB/c mice were injected s.c. on day 0 with 7000 4T1 cells and 10 days later, 1×10^9 pfu of AdCCL16 or Addl70-3 was injected intratumorally. The treatment was repeated 4 days later. Twelve mice were used in each group. Data are given as means +SD of tumor volume. ** $P < 0.01$ for (■) versus (◆).

To determine whether AdCCL16 treatment of the primary 4T1 tumor might affect metastatic dissemination and whether surgical removal of the primary tumor after treatment might synergize increasing survival, tumor-bearing mice were inoculated with AdCCL16 or the control virus at day 10 and day 14; in one group of mice, the primary tumor was surgically removed at day 19. At this time tumors size ranged between 3 and 6 mm in diameters and it is well known that mice with tumors of 2 mm have already disseminated metastases (Pulaski and Ostrand-Rosenberg, 1998). Part of the mice were followed for survival or sacrificed at day 29 and the number of lung metastasis scored by clonogenic assay.

Figure 4.19 schematizes the experimental protocol.

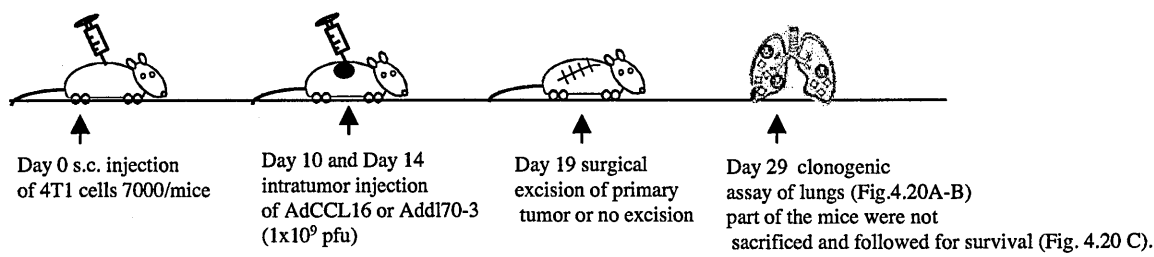


Figure 4.19 Scheme of the experiments presented in Fig 4.20

AdCCL16 reduced the number of lung metastases relative to the group that received the control virus (range 0-40 *vs* 50-5000; $p<0.001$), and when CCL16 treatment was combined with surgical removal of the primary tumor, 11 of 14 mice were metastases free and the remaining 3 mice had less than 10 metastases (range 0-8 *versus* 90-4000 in mice receiving Addl70-3 plus surgery; $p<0.001$) (Fig.4.20 A).

The abrogation of lung metastases obtained by combining CCL16 treatment with surgery was mainly T cell mediated since experiment performed in nu/nu mice showed a full metastatic capacity of 4T1 cells (Fig 4.20 B).

Moreover of a total of 30 mice that received AdCCL16 and surgery in three independent experiments, 19 were cured (63%; $p<0.001$), while all 24 mice treated with Addl70-3 and surgery succumbed (Fig 4.20 C). Challenge of surviving mice with 10^4 live 4T1 cells 90 days after primary tumor injection revealed protection in 12 of 19 mice (63%) indicating a long term immunity.

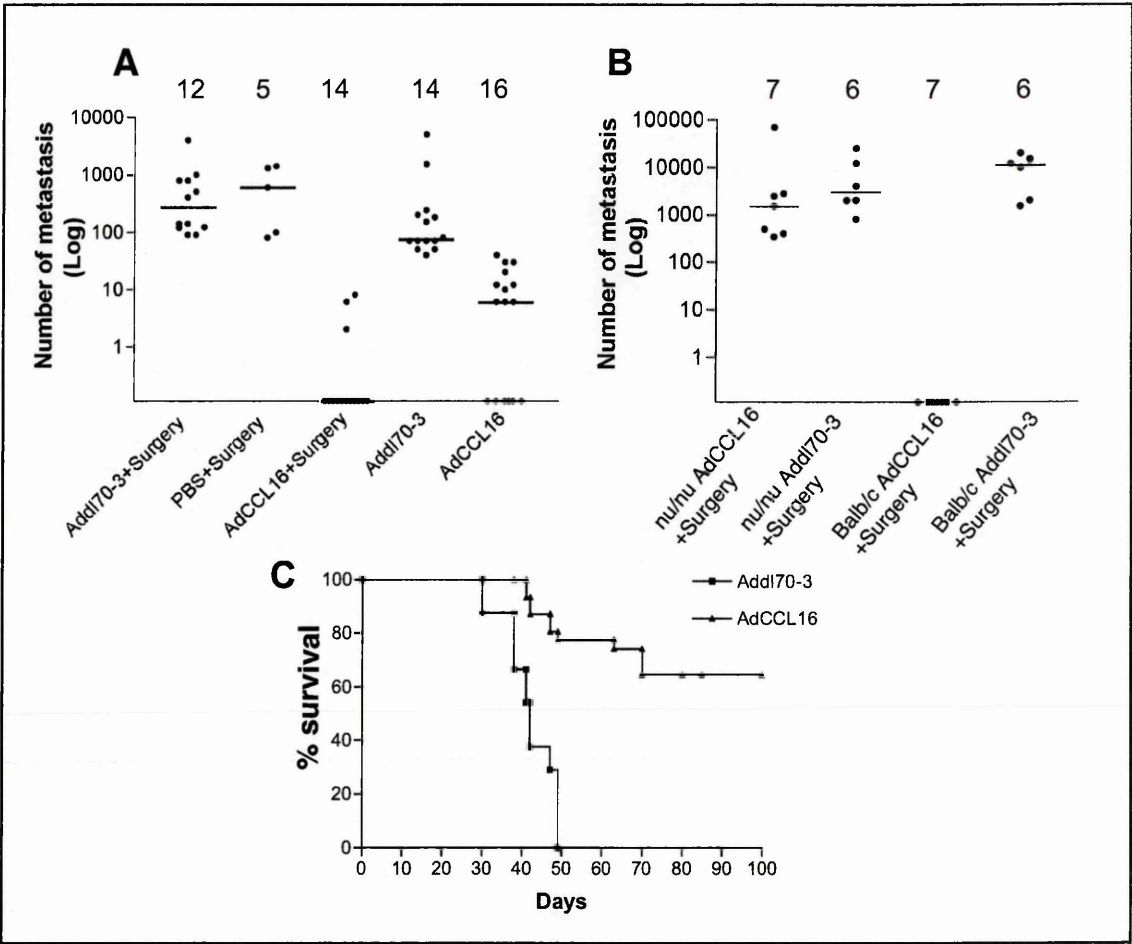


Figure 4.20 Effects of AdCCL16 treatment on metastatic spread of 4T1 tumors.

Experiments were performed as schematized in Figure 4.19. (A) Number of metastasis. Results are from two independent experiments (B) Role of T cells in the synergy between AdCCL16 treatment and surgical excision of primary tumor. Number of metastasis in nu/nu mice compared with wt BALB/c mice. For both A and B dot represents metastases from individual mice. Total number of treated mice is indicated on the top of each panels. Horizontal lines represent median. (C) Survival curve following AdCCL16 treatment and surgical excision of the primary tumor. Cumulative results of three independent experiments, (a total of 30 mice treated in the AdCCL16 treatment group and 24 in the Adl70-3 control group).

4.4 Discussion

Most experimental data obtained with transplantable tumors show that the best vaccine formulations effectively preimmunize mice against even a poorly or apparently non-immunogenic tumor challenge (Colombo and Forni, 1997) but that their efficacy declines if administered when the tumor overcomes a critical threshold and becomes clinically evident (Bocchia et al., 2000). Generally, only a minority of mice bearing established tumors has been cured, and only when the vaccine was administered in the first few days after tumor cell challenge (Cavallo et al., 1997). Similarly only few patient with established tumors display objective response, which in any event are only temporary (Parmiani et al., 2002).

Those results are not altogether surprising, considering that most of those patients have already been treated in various ways and no longer respond to conventional therapy. These considerations underline the need to investigate the use of molecules involved in the immune response in the context of existing tumors and moreover after removal of primary tumor (Bocchia et al., 2000).

CCL16 is remarkable *in vivo* with respect to rapid induction of systemic immunity and tumor rejection. Both features are important when dealing with transplantable murine carcinomas, since tumors can double or triple in size during the time required for vaccination to “instruct” the immune system. When transfected into TSA cells, CCL16 rendered these cells unable to form tumors *in vivo* due to rapid rejection by locally recruited monocytes, DC and CD8⁺ T cells. Most likely, CCL16 induces chemokines and cytokines by nearby leukocytes that provide the appropriate signal for massive inflammatory infiltration. In this context, it was found that a) CCL16 induces direct migration *in vitro* of CD4⁺ and CD8⁺ lymphocytes other than DC, at concentration of

1000-10 ng/ml, and (Chapter 3 Fig. 3.4 and 3.5) b) CCL16 induces potent CCL2/MCP-1 production by macrophages {Cappello, 2003 #95}, DC and T cells, (Chapter 3 Fig. 3.6).

The experimental setting of treatment established 3-4 mm tumors instead of injection of CCL16 transfected cell suspension necessitated delivery of CCL16 via an adenoviral vector. This size of TSA or MCA38 tumors exceeds that of any previous therapeutic attempt using immunological strategies (Cavallo et al., 1999). It was previously shown that TSA forms large highly invasive tumors of 2-3 mm in 7 days. At this time the tumor mass is well vascularized by self-induced vessel (Di Carlo et al., 1998).

In vivo, CCL16 production upon adenoviral delivery was quite similar to that of in vitro transduced TSA cells and the extent of ensuing tumor leukocyte infiltration was also similar. On the other hands use of stable in vitro-transfected cells (Giovarelli et al., 2000) or in vitro-AdCCL16 infected TSA cells (Fig 4.2) led to 75-85% tumor rejection versus 30% complete rejection when AdCCL16 was inoculated to established tumors (Fig 4.4). This observation is consistent with the difficulties for the immune response in eradicating large tumors, as underlined by the ability to reject normal tissue but not tumors expressing the same target antigen (Singh et al., 1992), and by the numerous mechanisms used by tumors to escape immune attack (Marincola et al., 2003). Indeed, mice bearing 1 day TSA established tumors are cured almost completely by repeated local administration of r-IL12. However the efficacy of this treatment disappear on 7 days tumors suggesting that at that time the tumor had grown to a point at which is no longer effectively controlled by the host's immune response (Cavallo et al., 1999).

In this chapter it was studied the mechanisms underling the rapid systemic immune response starting from the observed swelling of lymph nodes draining the AdCCL16 but not control Addl70-3-injected tumors. Node enlargement reflected the increased numbers of CD4⁺, CD8⁺ T lymphocytes and DC. Such DC stimulated a T cell clone specific for the

TSA TAA (Gri et al., 2002; Huang et al., 1996) (Fig. 4.16 A), suggesting the tumor origin of these cells.

Consistent with this finding, the lymphoid fraction, remaining after CD11c magnetic beads sorting, induced CTL activity against TSA and its TAA immunodominant epitope AH1 (Fig. 4.16 B).

T lymphocytes from LN draining AdCCL16 but not control TSA-treated tumors produced IFN- γ (Fig. 4.14), an effect that is not a direct consequence of CCL16 expression since CD4⁺ and CD8⁺ purified T cells in vitro did not produce IFN- γ when co incubated for 24 or 48h with recombinant CCL16.

The finding that AdCCL16 treatment performed in animal depleted of T cells as well as in IFN- γ KO mice loose its effectiveness suggest a role for T cells and the IFN- γ produced upon their activation in mediating AdCCL16 antitumor effect (Fig. 4.17).

IFN- γ plays an important role in immune-mediated tumor destruction. Its antitumor effects are mediated by: 1) the ability to stimulate cell mediated immune responses; 2) the induction of DC activities necessary to support Th1 development and cell mediated immune responses; 3) the inhibition of tumor neoangiogenesis. Moreover, IFN- γ production at tumor site stimulates the expression of gene products that when induced in tumor cells themselves or in leukocytes infiltrating treated tumors may be responsible for a direct anti-tumor activity. These include the expression of enzyme systems that generate reactive oxygen and nitrogen products by a variety of myeloid cell types. Indeed, IFN- γ , can stimulate endothelial cells, epithelial cells, T cells and macrophages to synthesize iNOS and to produce NO, which induces tumor cytotoxicity [reviewed in ref. (Tannenbaum and Hamilton, 2000)]. To this extent IFN- γ production was found to be produced by CD8 T cell lymphocytes infiltrating TSA treated with AdCCL16 (Fig. 4.8) and in T cells from LN draining the same treated tumors (Fig. 4.14).

The robust and specific immune response against the tumors was therapeutic for only ~30% of mice. An effort to improve this rate refuted to increase to number of AdCCL16 injection because of the vector immunogenicity, rather it focused on application of AdCCL16 in a more clinically relevant context that generally requires surgical removal of primary tumor since the tumor lethality usually stems from the relatively small number of cells that remain after surgical excision, radiotherapy and chemotherapy.

Although the TSA carcinoma has metastatic capacity, this model is cumbersome (Cavallo et al., 1999). Perhaps the most compelling model for studying spontaneous metastases, at least on a BALB/c background, is the 4T1 mammary carcinoma (Pulaski and Ostrand-Rosenberg, 1998), since metastases spread immediately after subcutaneous tumor injection and the process is not impaired by removal of primary tumor (Pulaski et al., 2000).

The 4T1 tumor model has been used extensively by Ostrand-Rosenberg and colleagues to study the therapeutic potential of cellular vaccines composed of various immunomodulatory molecules on metastatic spread (Ostrand-Rosenberg et al., 1998; Pulaski and Ostrand-Rosenberg, 1998; Pulaski et al., 2000).

Vaccination with tumor cell transfected with MHC class II and B7.1 without removal of the primary tumor reduced the number of metastases but had no impact on survival (Pulaski and Ostrand-Rosenberg, 1998). Also, a treatment with an immunostimulator as mB7-2 Ig fusion protein combined with the angiogenesis inhibitor SU668, started as earlier as 3 days after tumor injection significantly reduced tumor growth and number of lung metastases but did not achieved any cure (Huang et al., 2002b).

A critical issue is the size of the tumor at the time of vaccination, since tumors exceeding 4 mm in diameter already have a large metastatic load precluding successful treatment (Pulaski et al., 2000). Surgical removal of the primary tumor as the first line of treatment in the 4T1 model was not sufficient to arrest metastases-induced death, but did change the setting in which a vaccine is called to act against residual disease (numerous small

metastases). In 4T1 tumor-bearing mice, removal of primary tumors followed by repeated vaccination with MHCII- and B7-1-co-transfected cells plus cells transfected with SEB superantigen encoding gene, significantly extended survival time but did not cured the mice (Pulaski et al., 2000).

A more effective strategy might involve the treatment of primary tumors before surgery, an approach that might reduce the time for priming the host against its tumor. In fact, AdCCL16 injected into tumor nodules twice at days 10 and 14, followed by surgical removal at day 19, cured 19 of 30 mice (63%) and reduced the number of lung metastases in mice sacrificed for clonogenic assay (3 mice with less than 10 metastases and 11 free of metastases) (Fig.4.20 A-B). A similar approach using a combination of adenoviruses encoding thymidine kinase (TK), IL-2 and GM-CSF reduced the number of lung metastases and prolonged survival of 6 mice of 20 in a follow-up of only 35 days (Majumdar et al., 2000). A rough comparison of those data and the ones presented in this chapter suggests that CCL16 is more effective than such multiple combinations.

Better results (cure rate of 90%) has been obtained only in one study through a more intensive treatment protocol such as surgery followed by vaccination with syngeneic tumor cells mixed with bystander cells, and finally donor lymphocyte infusion of mice transplanted with allogeneic stem cells under non myeloablative conditions (Luznik et al., 2003).

A curative rate of 60% was also obtained by surgical excision of primary 4T1 tumor injected into STAT6^{-/-} mice (Ostrand-Rosenberg et al., 2002; Ostrand-Rosenberg et al., 2000). While the lack of STAT6 signaling protects these mice from IL-13- mediated immunosuppression (Jensen et al., 2003; Ostrand-Rosenberg et al., 2002) a possibility is the recognition of tumor- associated STAT6 as a foreign antigen (Jensen et al., 2003).

It is unclear why the effectiveness of this AdCCL16 treatment on primary TSA and 4T1 tumor regression is limited to a small number of mice. In the 4T1 model, the lack of

STAT6 has been shown to impair development of metastases but was not sufficient to induce primary tumor rejection (Ostrand-Rosenberg et al., 2002). In addition to NKT cell produced IL-13 immunosuppression of an unknown target cell (Terabe et al., 2000), other immunosuppressive cytokines such as IL-10, PGE2 and TGF- β produced by tumors or infiltrating leukocytes impair function and survival of tumor-associated APC (Vicari AP, 2002). In this context strategies aimed neutralizing IL-10 in the TSA model (Vicari et al., 2002) or TGF- β in the 4T1 model have led to the functional restoration of tumor-infiltrating APC (Kobie et al., 2003).

Together, the results obtained from this area of the study with CCL16 given alone suggest that the therapeutic potential of this chemokine might be augmented by strategies aimed to further improve antigen-presenting functions of DC recruited at the tumor site.

CHAPTER 5: AdCCL16 POTENTLY SYNERGIZES WITH A MICROBIAL STIMULI (CpG-ODN) AND BLOCK OF THE IL10 RECEPTOR

5.1 Introduction

Although tumors are capable of autonomous growth, their progression is highly influenced by their stromal component (Coussens and Werb, 2002). Thus, the tendency of novel therapeutic approaches is to target both the tumor cells and their embedding stroma. The extracellular matrix, the blood vessel fibroblasts and tumor infiltrating leukocytes such as macrophage, in addition to their structural role, may exert either inhibiting or promoting effects on tumor growth (Gordon, 2003; Mantovani et al., 2002). Two distinct activation states of macrophages, which are among the most abundant leukocytes infiltrating mouse and human tumors, have been described: conventionally activated M1 macrophages produce high levels of inducible nitric oxide synthase (iNOS), IL-12 and TNF- α whereas the alternatively activated M2 macrophages produce arginase, IL-10, TGF- β and PGE2. M1 macrophages are extremely potent effector cells that kill tumor via nitric oxide (NO) and TNF- α (Klimp et al., 2002), while M2 macrophages promote angiogenesis, tissue remodeling and limit Th1 immune responses (Mantovani et al., 2002). Macrophages with M2 phenotype have been isolated from mouse and human tumors and shown to suppress T cell activation and proliferation through IL-10 and TGF- β (Balkwill and Mantovani, 2001). Thus, although tumor-infiltrating macrophages are potentially able to induce an anti-tumor innate response, their activation state is modulated in the tumor micro-environment and they may be redirected to exert a tumor growth promoting effect. Similarly to macrophages, TIDC have been shown to be defective in their ability to produce pro-inflammatory cytokines such as IL-12 and to induce innate IFN- γ production or adaptive Th-1 responses.

In order to initiate and maintain an effective anti-tumor response after TAA uptake, DC should migrate to draining lymph nodes and prime T cells. This process is triggered by an activation-driven maturation process of DC characterized by up-regulation of costimulatory molecules (CD40, CD80, CD86), a switch in the chemokine receptor repertoire, and production of immunomodulatory cytokines (IL-12, IFN- α) necessary for the generation of cytotoxic T cells (CTL) (Chiodoni et al., 1999). However, immunosuppressive cytokines such as IL10, TGF- β , PGE2 and VEGF interfere with DC maturation and migration, hampering the anti-tumor response.

A possible immunotherapeutic approach would be to target the tumor escape mechanisms that paralyze the macrophage/DC tumor infiltrate and prevent both innate and adaptive anti-tumor responses. Indeed, repeated treatments with a combination of a microbial stimulus (a TLR9 ligand, CpG oligonucleotide) and an antibody blocking the IL10R were able to revert the functional paralysis of tumor-infiltrating DC and to re-establish their ability to produce IL-12 (Vicari et al., 2002).

In chapter 4 it was found that the delivery via adenoviral vector of the chemokine CCL16 in established TSA mammary carcinomas induced a specific systemic anti-tumor response and a massive accumulation of leukocytes (T cells, macrophages and DC) at the tumor site. However, a complete tumor rejection was not accomplished, possibly due to the lack of proper activation of the TIDC.

In this chapter it was investigated whether the leukocyte infiltrate promoted at the tumor site by CCL16 expression was paralyzed by the tumor microenvironment. Moreover it was investigated whether combination with a microbial stimuli CpG and block of the immunosuppressive cytokine, IL-10, could revert such paralysis synergizing with CCL16 in inducing rejection of large primary tumors.

5.2 Aims of the chapter

In this chapter the issues introduced above were investigated by:

- evaluating the phenotype of macrophages and DC accumulating in AdCCL16 treated tumors;
- analyzing whether CCL16 synergizes with local CpG treatment and systemic block of the IL-10 receptor in promoting rejection of TSA, MCA38 and 4T1 primary tumors;
- characterizing the innate response that follows the treatment;
- evaluating the relative contribution of the innate and adaptive responses in tumor rejection.

5.3 Results

5.3.1 CCL16 potently synergizes with CpG and anti-IL10R to reject large tumors

It was tested whether adenoviral vector delivery of CCL16 into large established tumors in combination with a single injection of CpG and anti-IL-10R synergized to reject larger primary tumors. The same three tumor model, TSA, 4T1 and MCA38 used in chapter 4 to test antitumor effect of AdCCL16 given alone were used to investigate the combinational regime. Tumors of 5 mm or larger were injected with AdCCL16 and 36 h later with CpG while anti-IL10R was given systemically. When mice were treated with CCL16 in combination with either anti-IL10R or CpG less than 20% of mice rejected the tumor. Similarly, control virus Add170-3 combined with CpG and anti-IL10R showed little therapeutic effect (20 to 30% tumor rejection). In contrast, when CCL16 was combined with CpG and anti-IL10R, up to 90% of the mice rejected established TSA tumors and 74% the MCA38 tumors and 60% the 4T1 tumors (Fig 5.1 A-C) (all $p < 0.001$ vs Add170-3+CpG+anti-IL10R control groups). Mice rendered tumor-free after a single treatment

with CCL16, CpG, and anti-IL10R acquired long-term immunity and rejected a challenge with live tumor cells 100 days later.

In chapter 4 (Fig. 4) it was confirmed the metastatic potential of 4T1 cells that disseminate to several distant organs as soon as the primary tumor reach 2 mm in diameter (Pulaski and Ostrand-Rosenberg, 1998).

In order to determine whether the combination treatment affected the metastatic dissemination of 4T1 mammary carcinoma, 4T1 nodules were treated with AdCCL16 alone or combined with CpG plus anti-IL10R. Mice were sacrificed at day 32 after tumor inoculation and scored for lung metastases by a clonogenic assay. While treatment with AdCCL16 significantly reduced the number of lung metastases (range 12000-160000 *vs.* 1000-23600 of Addl70-3 *vs* AdCCL16; $p=0,02$), the additional treatment with CpG plus anti-IL10R completely prevented metastases in 7 mice out of 14 and reduced the number to less than 10 in the remaining 7 mice (Fig 5.1 D) (range 1000-2000 *vs* 0-12 between AdCCL16 or Addl70-3 combined with CpG+antiIL10R; $p<0,001$).

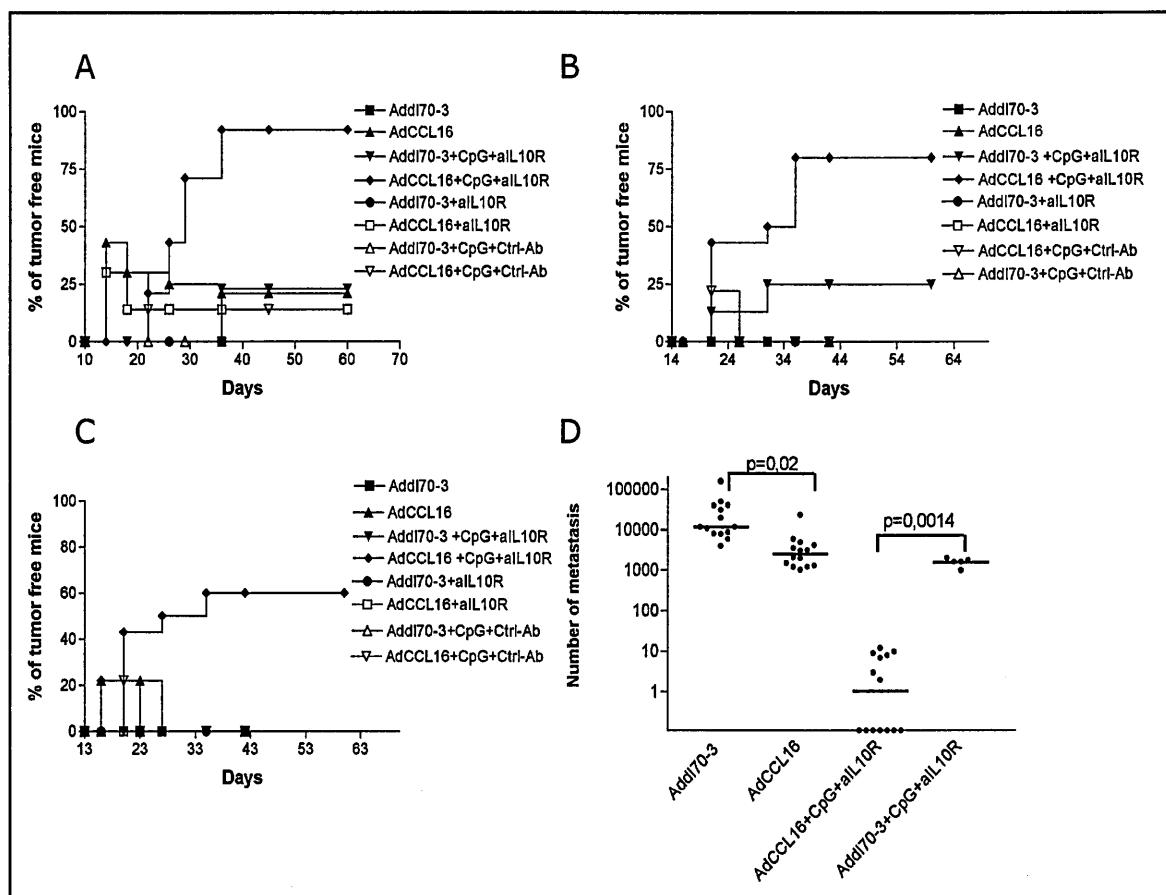


Figure 5.1 Combination of CCL16, CpG and anti-IL10R induces rejection of pre-existing tumors.

Mice bearing TSA (A), MCA38 (B), or 4T1 (C) tumors were injected intratumorally with 1×10^9 pfu of AdCCL16 or control Addl70-3 and 36 h later left untreated or treated with anti-IL10R (200 μ g) intraperitoneally and CpG intratumorally (5 μ g). D) Effects of the combinatory treatment on the metastatic spread of 4T1 tumors. Mice bearing 4T1 tumors were intratumorally injected with 1×10^9 pfu of AdCCL16 or Addl70-3 and 36 h later mice were left untreated or treated with CpG plus anti-IL10R. Metastases were evaluated in the lung 32 days after tumor inoculation and results expressed per individual mice. Cumulative results of two independent experiments each performed with 7-8 mice are shown.

5.3.2 The early anti-tumor effect of CCL16, CpG, and anti-IL10R combination is the result of a rapid innate inflammatory response

Tumor-bearing mice treated with Addl70-3 or AdCCL16 combined or not with CpG and anti-IL10R were sacrificed for macroscopic evaluation of the tumor area 6 and 16 h post-treatment. Although CpG plus anti-IL10R combined with control Addl70-3 showed partial necrosis, only the combination with AdCCL16 induced tumor shrinkage associated with a massive necrosis that was mostly colliquative as indicated by blood extravasion already present 6 h post-treatment (Fig 5.2).

Microscopically, tumors from the animals treated with Addl70-3 and CpG plus anti-IL10R showed discrete areas of necrosis, while in the nodules treated with AdCCL16 and the same combination the necrotic area extended to almost the entire tumor mass (Fig 5.3).

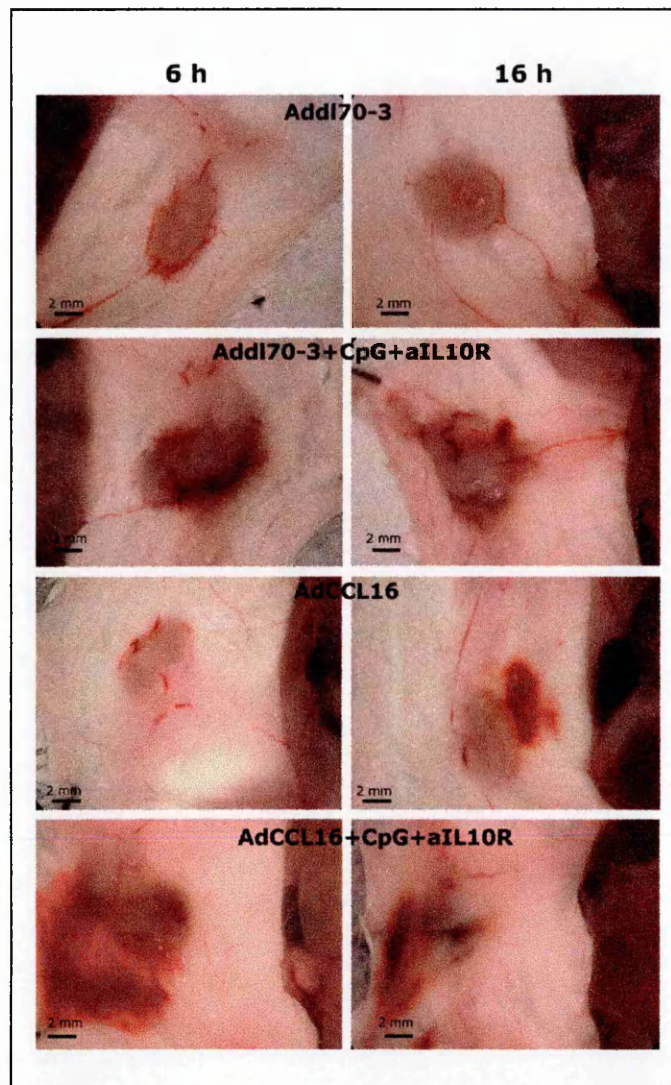


Figure 5.2 Macroscopic evaluation of treated tumors.

Mice bearing TSA tumors were injected intratumorally with AdCCL16 or control Addl70-3 and 36 h later treated with CpG plus anti-IL10R or PBS as control. 6 and 16 h later skin was opened and tumors photographed unfixed to show size, gross vascularization and necrosis. While injection of control Addl70-3 allowed progressive growth of well-vascularized tumors, the addition of CpG and anti-IL10R provoked necrosis in part of the tumor while other grow normally. Injection of AdCCL16 induced small hemorrhagic area at 16 h while its combination with CpG and anti-IL10R lead to impressive induction of necrosis coupled with large hemorrhagic area already at 6 h after treatment. Ten hours later, most of the tumor area is flat, the nodule almost dissolved and the hemorrhagic area already partially reabsorbed.

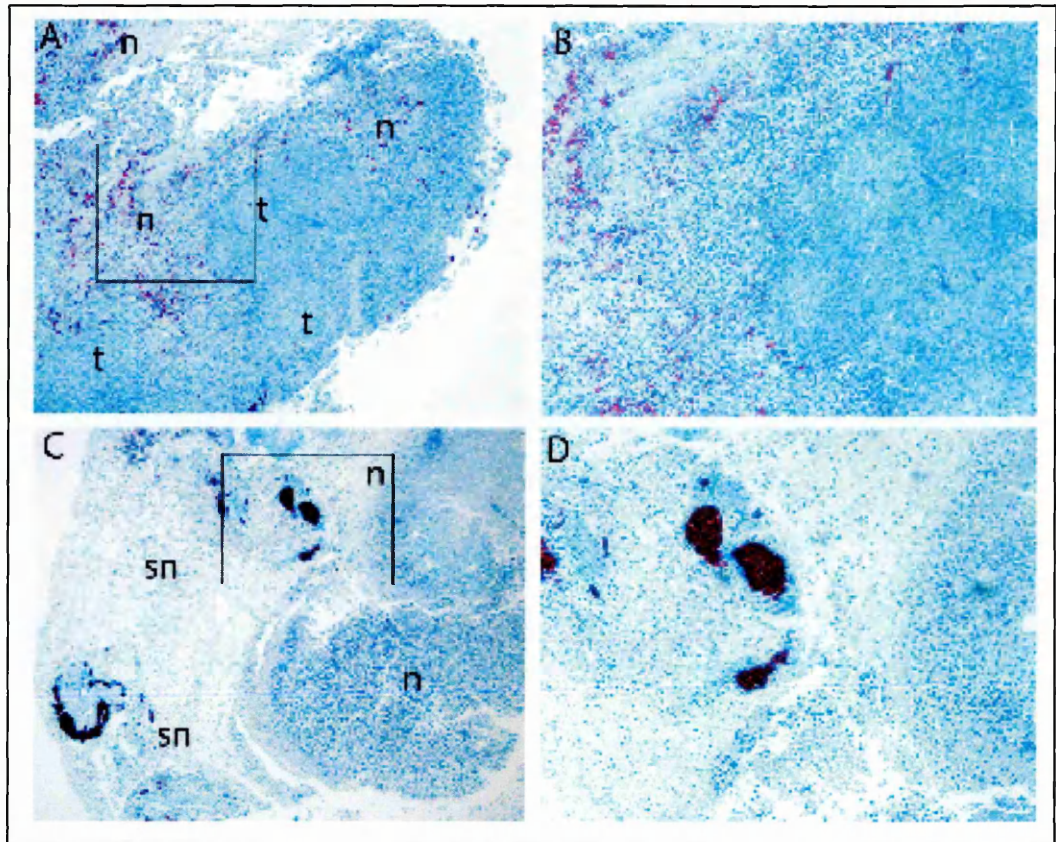


Figure 5.3 Histological evaluation of treated TSA tumors.

Representative sections of treated TSA tumors were stained with Masson's trichrome. Pre-established TSA tumors were treated with Addl70-3 (A-B) or AdCCL16 (C-D) together with CpG and anti-IL10R. Mice were sacrificed 16 h later. Tumors treated with AdCCL16, CpG and anti-IL10R demonstrated massive necrosis in both tumors (n) and stroma (sn), consistent with hemorrhagic necrosis. On the contrary, Addl70-3 combined with CpG and anti-IL10R showed extended areas of necrosis alternated with large areas of live tumor tissue (t) (x40). B and D are enlargement of boxed area (x100).

To gain insight on the mechanism of the synergistic effect of CCL16 and CpG plus anti-IL10R, the cellular infiltrate recruited by CCL16 expression at the tumor site was analyzed 36 h after AdCCL16 treatment.

Alteration of the function of tumor infiltrating macrophages has been described and includes production of IL-10, defective production of reactive nitrogen, down modulation of inflammatory cytokines such as TNF- α and IL-12 and impaired tumor cytotoxicity. Indeed, TSA tumors treated with a single injection of AdCCL16 showed a significantly higher number of F480+ macrophages and CD11c+ DC than tumors treated with control adenovector or PBS (Fig. 5.4 A). However, macrophages infiltrating CCL16-treated tumors produced IL-10 as suggested by staining co-localization, but not detectable level of TNF- α or IL-12, consistent with an M2 phenotype (Mantovani et al., 2002) (Fig. 5.5 A-B). In tumors treated with the control adenovector, macrophages, although less numerous (Fig. 5.4), also expressed IL-10 (Fig. 5.5 C-D), indicating that, although CCL16 is able to promote macrophages infiltration, it is unable to prevented their alternative activation into a M2 phenotype once they have been recruited into the tumor microenvironment. In agreement with this conclusion the observation that despite the abundant infiltrate only minimal necrotic foci were found in CCL16 treated tumors strongly indicating an inability of the macrophage infiltrate to initiate massive tumor destruction.

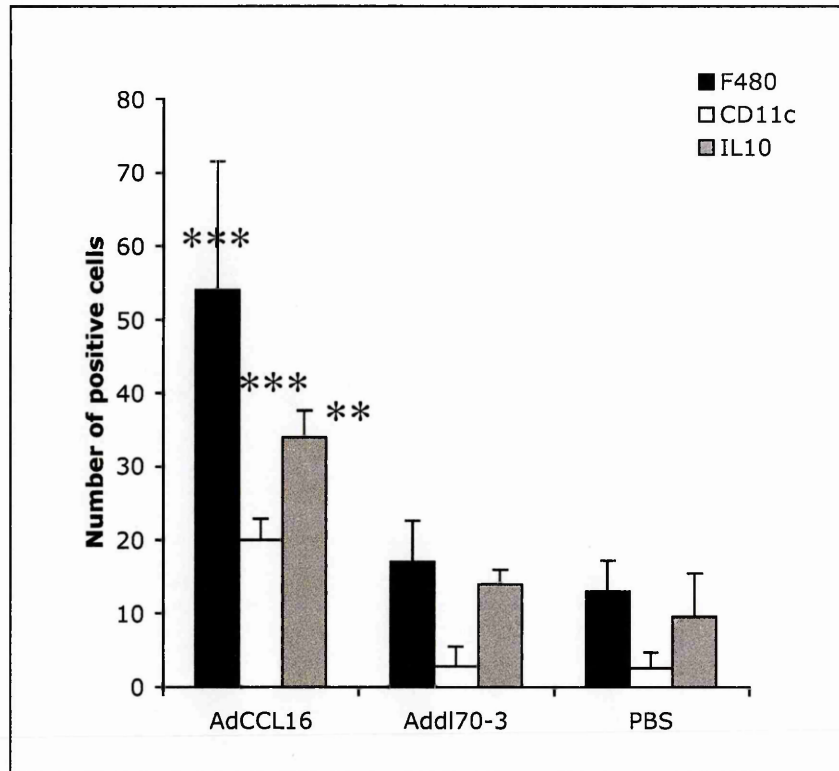


Figure 5.4 Immunohistochemical analysis of TSA tumors after AdCCL16 or Addl70-3 treatment.

TSA tumors were injected with AdCCL16 or with Addl70-3 or with PBS and processed for immunohistochemistry analysis 36 h later. Cryostat sections were stained for the macrophage marker F4/80, the dendritic cell marker CD11c and for IL-10 expression. Cumulative data are presented as bars (mean \pm SD of positive cells counted in 10 randomly chosen field from three tumors in each group). A significantly higher number of F4/80, CD11c and IL-10 positive cells were detected in AdCCL16 treated tumors as compared with tumors treated with the empty vector Addl70-3 or PBS. *** $P=0,001$, ** $P=0,01$.

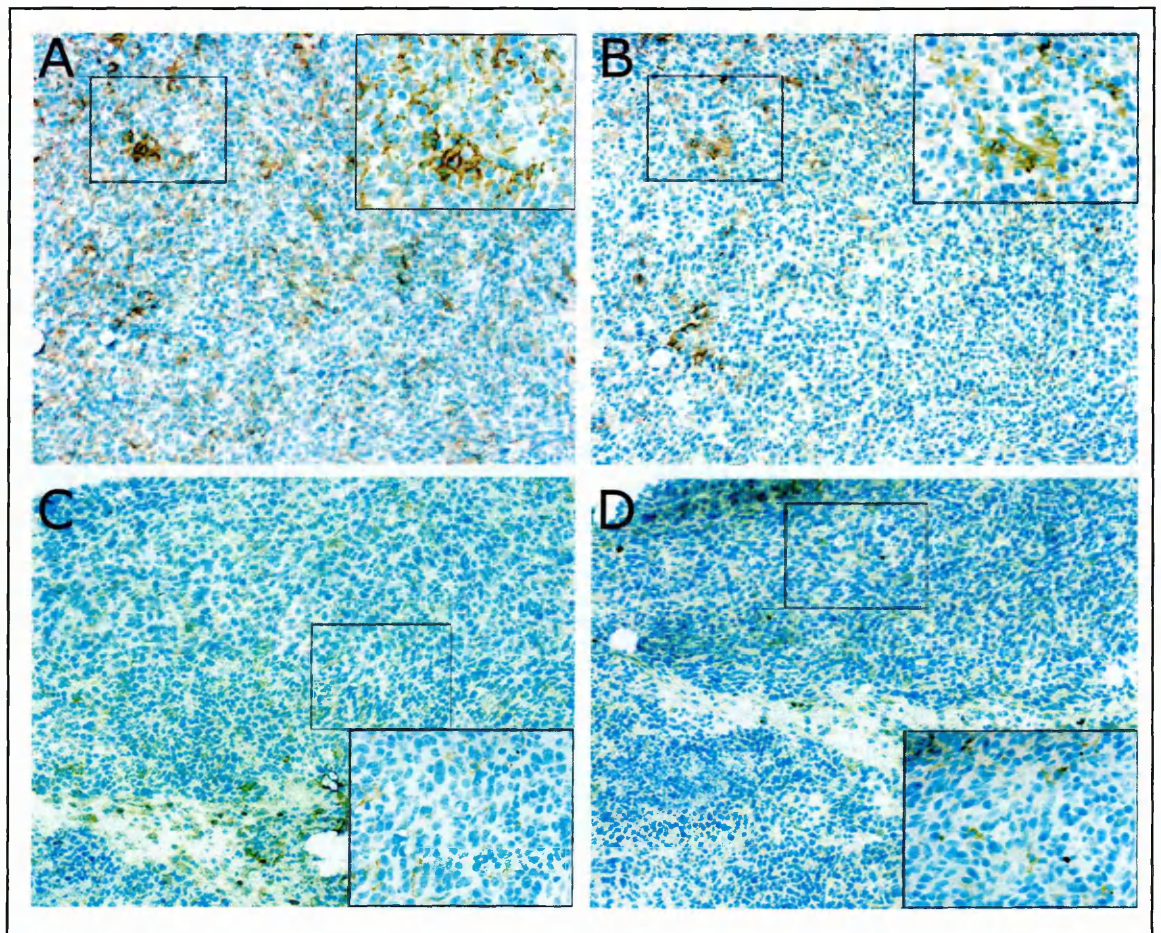


Figure 5.5 Macrophages infiltrating TSA tumors produce IL-10.

TSA tumors were injected with AdCCL16 (A-B) or with Addl70-3 (C-D) and processed for immunohistochemistry analysis 36 h later. Serials cryostat sections were stained for the macrophage marker F4/80 and for IL-10 expression. Macrophages (F4/80) (A-C) colocalize with IL-10 (B-D) (x100) (the insert show magnification of the boxed area, x400).

PMN leukocytes have been shown to participate in immunosurveillance against tumors where they have been found responsible for the hemorrhages and thrombosis associated with tumor necrosis. PMN produce several cytotoxic mediators including hypochlorous acid (HOCl), which is a membrane-perforating agent, TNF- α , IL-1 β and IFNs (Di Carlo et al., 2001). To understand whether PMN could be responsible of the potent necrotic reaction exerted by combination treatment, serial tissue sections of TSA treated-tumors where stained with GR-1 and F480 Abs to identify PMN and macrophages, respectively. As shown in Fig. 5.6 the abundant macrophagic infiltrate localized at the edge and in the inner part of the tumor inside the area of necrosis while PMN, present in a much lower number, were found predominantly at the edge of the tumor. This result suggests that macrophages are the main subsets of leukocytes responsible for the necrosis associated with the early inflammatory response.

The addition of CpG plus anti-IL10R to the CCL16 treatment drastically changed the activity of tumor-associated macrophages that became able to produce TNF- α and IL-12 (Fig. 5.7 A-D).

One of the most potent cytostatic molecules released by activated macrophages is nitric oxide (NO) (Klimp et al., 2002). AdCCL16 induced three times more NO than Add170-3 when combined with CpG plus antiIL10R (Fig. 5.7 E). Together these results indicated a role of CCL16 in recruiting macrophages and of CpG plus antiIL10R in restoring their ability to exert an anti-tumor activity throughout mechanisms of the innate response.

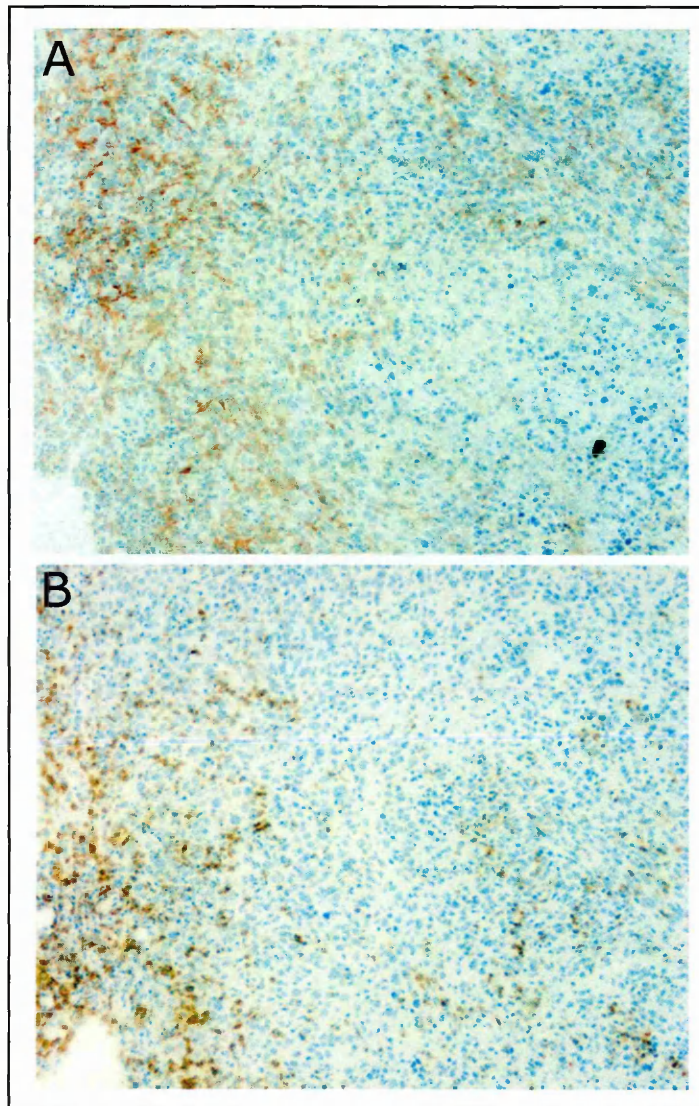


Figure 5.6 Localization of macrophages and PMN in treated tumors.

Pre-established TSA tumors were treated with AdCCL16 together with CpG and anti-IL10R. Mice were sacrificed 16 h later. Serials cryostat sections were stained for the macrophage marker F4/80 (A) and for the PMN marker GR-1 (B).

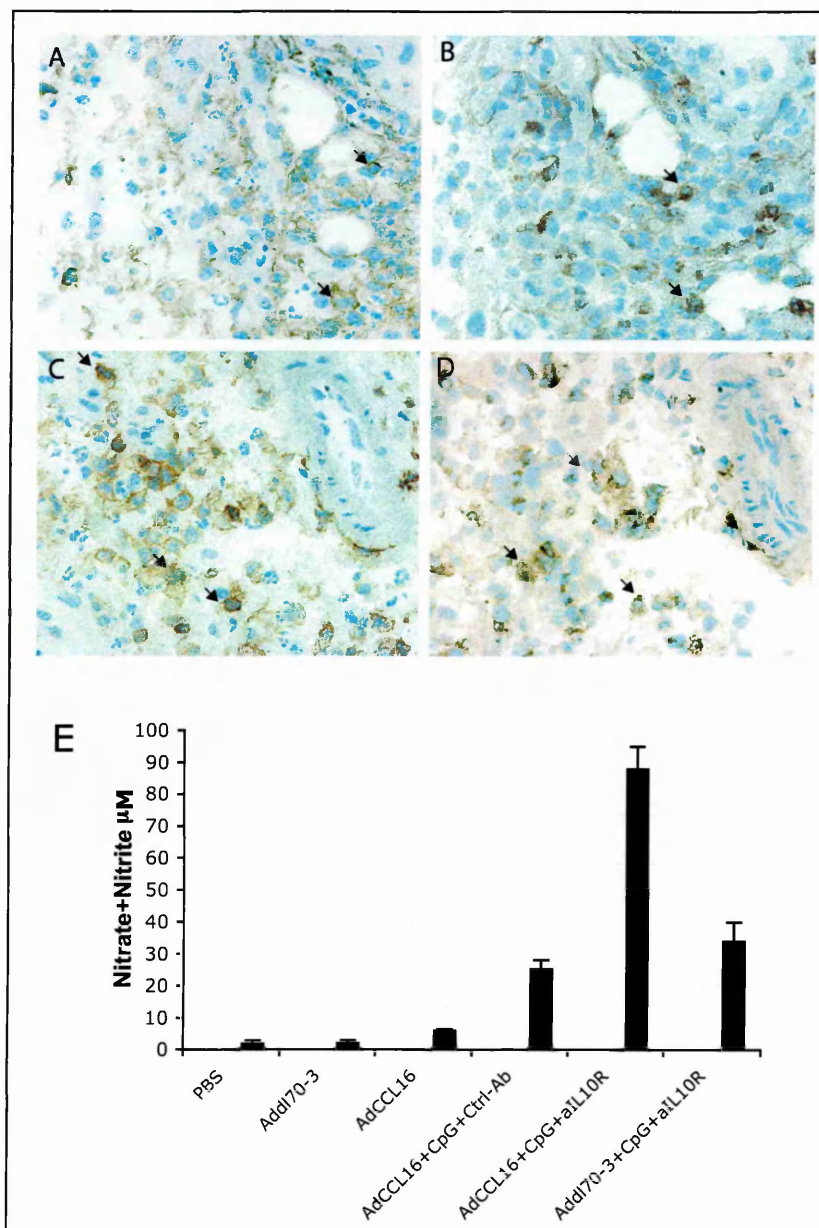


Figure 5.7 Macrophages infiltrating treated tumors express TNF- α , IL-12 and Nitric Oxide.

TSA tumors were injected with AdCCL16 and 36 h later with CpG and anti-IL10R. Tumors were collected for immunohistochemical analysis 6 h later. Macrophages (F4/80) (A, C) co-localized in serial sections with TNF- α (B) and IL-12 (D) (x400). Arrows indicate co-localization of stained cells in serial sections E) Production of nitric oxide in response to the indicated treatments. Tumors were collected for analysis 4 h after treatment (mean \pm SD from five tumors per group; one of two independent experiments is shown).

5.3.3 Induction of tumor specific adaptive response by combination treatment.

To confirm the primary finding that specific antitumor immunity was induced after combination treatment, CTL activity in tumor bearing mice after treatment was evaluated. As shown in Fig. 5.8 TSA tumor-bearing treated with AdCCL16, CpG and anti-IL10R developed tumor specific systemic CTL activity as early as 7 days post-treatment.

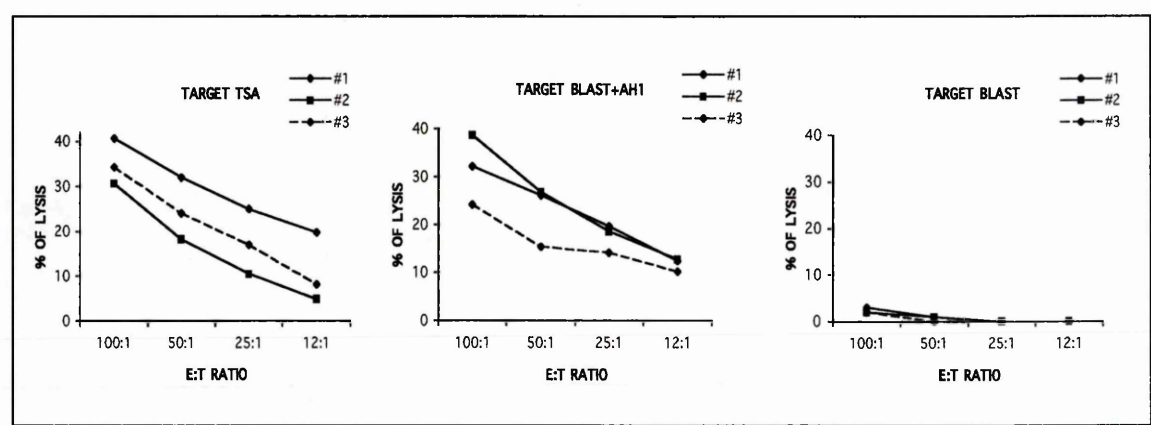


Figure 5.8 Induction of CTL in treated mice.

TSA tumors were injected with AdCCL16 and 36 h later with CpG and anti-IL10R. Splenocytes collected 7 days after treatment were *in vitro* restimulated with the AH1 antigenic peptide of TSA and cytotoxicity measured against TSA tumor cells or blast cells loaded or not with the AH1 peptide. Results are representative of two independent experiments, each performed with three mice.

Evidences that the *in vitro* specific cytolysis was relevant *in vivo* came from Winn Assay experiments. Lymphocytes freshly isolated from LNs and spleens of mice treated with AdCCL16 or Add170-3 combined with CpG plus anti-IL10R were mixed to a 50:1 ratio with live TSA cells and injected in naïve mice. Based on the percentage of mice free of tumors it appears that AdCCL16 combined with CpG plus anti-IL10R leads to a better priming of T lymphocytes (Fig 5.9).

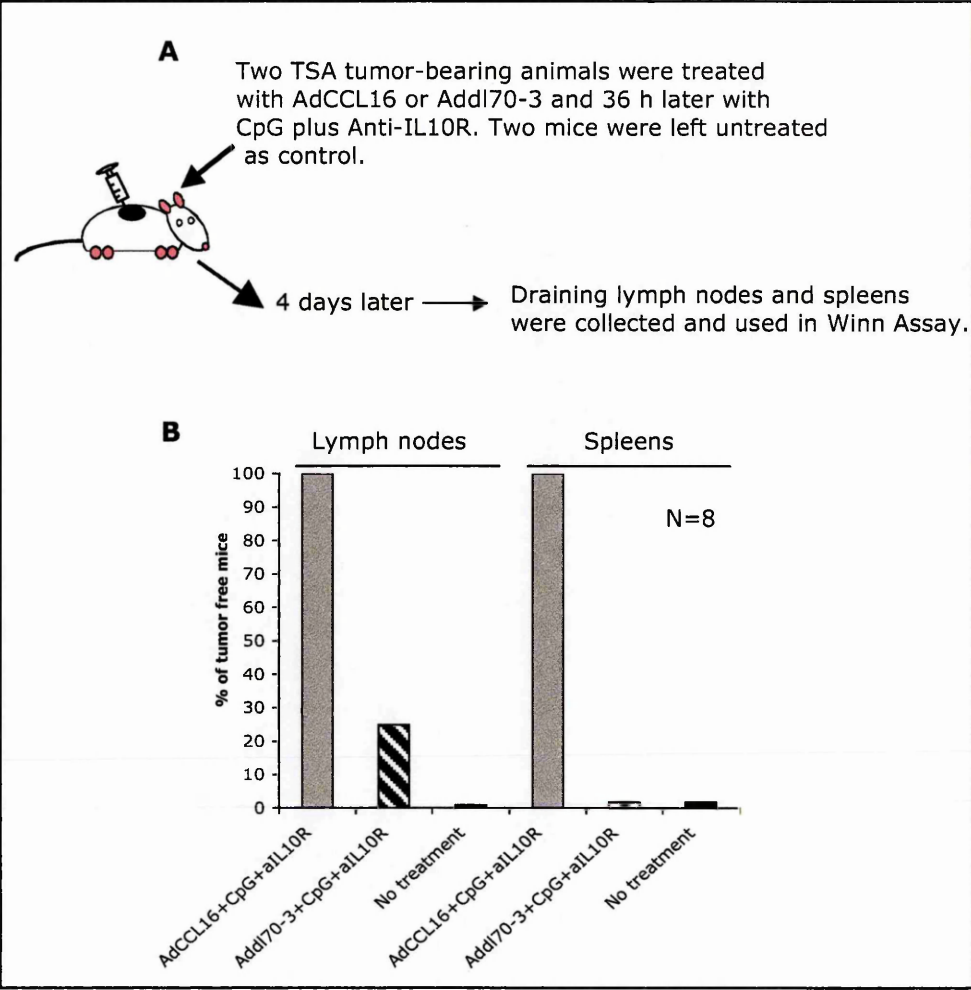


Figure 5.9 Winn assay for protection of TSA tumor bearing mice by lymphocytes from treated mice.

A) Scheme of the experiment. TSA tumor bearing mice (n=2) were treated with AdCCL16 or Addl70-3 combined with CpG plus anti-IL10R or left untreated. Four days later, draining lymph nodes and spleens were collected and lymphocytes mixed with live TSA cells to a ratio of 50:1 (T cells: tumor cells) and injected s.c. in BALB/c mice. B) Lymphocytes from each DLN and spleen were transferred to 4 mice for a total of 8 mice per group. The percentage of mice free of tumor is given at day 90.

5.3.4 Innate and adaptive responses participate in the eradication of established large tumors.

Next it was tested whether innate resistance was sufficient or adaptive immunity was also required for complete tumor rejection. When TSA tumor-bearing Rag-KO mice, which are deficient of T and B cells, were treated with AdCCL16, CpG plus anti-IL10R, only 28% of them completely rejected the tumors (Fig. 5.10 A).

Antibody-mediated depletion of either CD4 or CD8 T cells starting one week before tumor challenge also reduced to 50% the number of BALB/c mice rejecting TSA tumors (Fig. 5.10 A).

To better dissect the role of CD4 and CD8 T cells in the effector phase of the rejection process, they were depleted by antibody treatment of the mice 5 days post AdCCL16 and CpG plus anti-IL10R treatment. Late CD8 T cell depletion still reduced the rejection rate to 37% compared to 85% in the non-depleted group. Unlike CD8 T cells, late CD4 T cell depletion did not affect the rejection rate, suggesting that CD4 T cells had a role during the priming rather than the effector phase whereas CD8 T cells were required in both phases (Fig. 5.10 B).

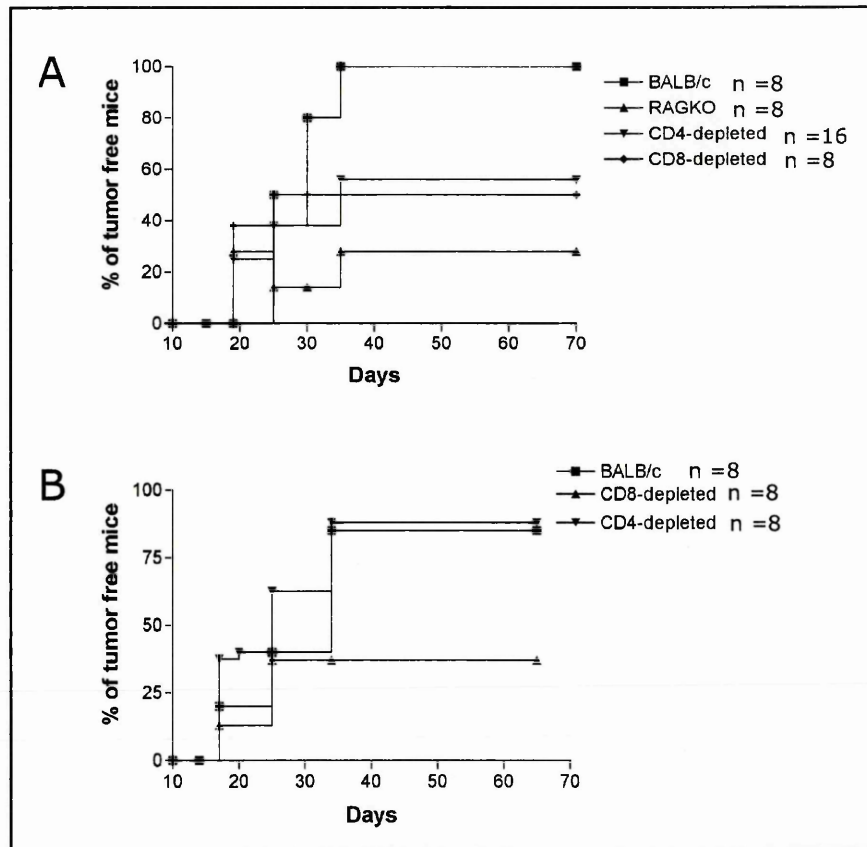


Figure 5.10 Role of CD4 and CD8 T cells in CCL16, CpG and anti-IL10R-induced tumor rejection.

TSA tumors were injected with AdCCL16 and 36 h later with CpG and anti-IL10R. A) Tumor rejection was monitored in mice depleted of CD4 or CD8 T cells prior to treatment as well as in RAG-KO mice. B) Tumor rejection was monitored in mice in which CD4 and CD8 T cells were depleted starting five days after treatment. The number of mice treated is indicated in the figure.

Together, the above results unambiguously indicated that complete eradication of the tumors required the participation of T cells and suggested that both innate and adaptive responses were involved in tumor eradication. However, both conventional T cells as well as non-conventional T cell subsets such as NKT and $T\gamma/\delta$ cells can be recruited in a non-antigen specific fashion in amplifying the innate response, for example by producing tumor-inhibiting cytokines such as IFN- γ . Thus, it was important to test whether mice in which T cells were present but were unable to mount a tumor-specific adaptive response were able to eradicate the tumors when treated with the combination protocol. This was accomplished by using bone-marrow (BM) chimeric mice in which only the adaptive response was impaired because of H-2 mismatch. Irradiated CB6F1 mice reconstituted with BM cells from either B6 or BALB/c were injected with TSA tumor cells (H-2d). CTL were able to recognize H-2d-presented TSA antigens in BALB/c>CB6 but not in B6>CB6 chimera. Whereas 92% of AdCCL16, CpG plus anti-IL10R treated BALB/c>CB6 mice rejected the TSA tumors, only 26% of B6>CB6 did so (Fig. 5.11A). The innate response measured as NO production was comparable in the two groups of treated mice (Fig. 5.11B).

These results strongly indicate that in mice in which the innate response failed to completely eradicate the primary tumor, adaptive T cell responses, in particular CD8 T cells, were essential to control local recurrence.

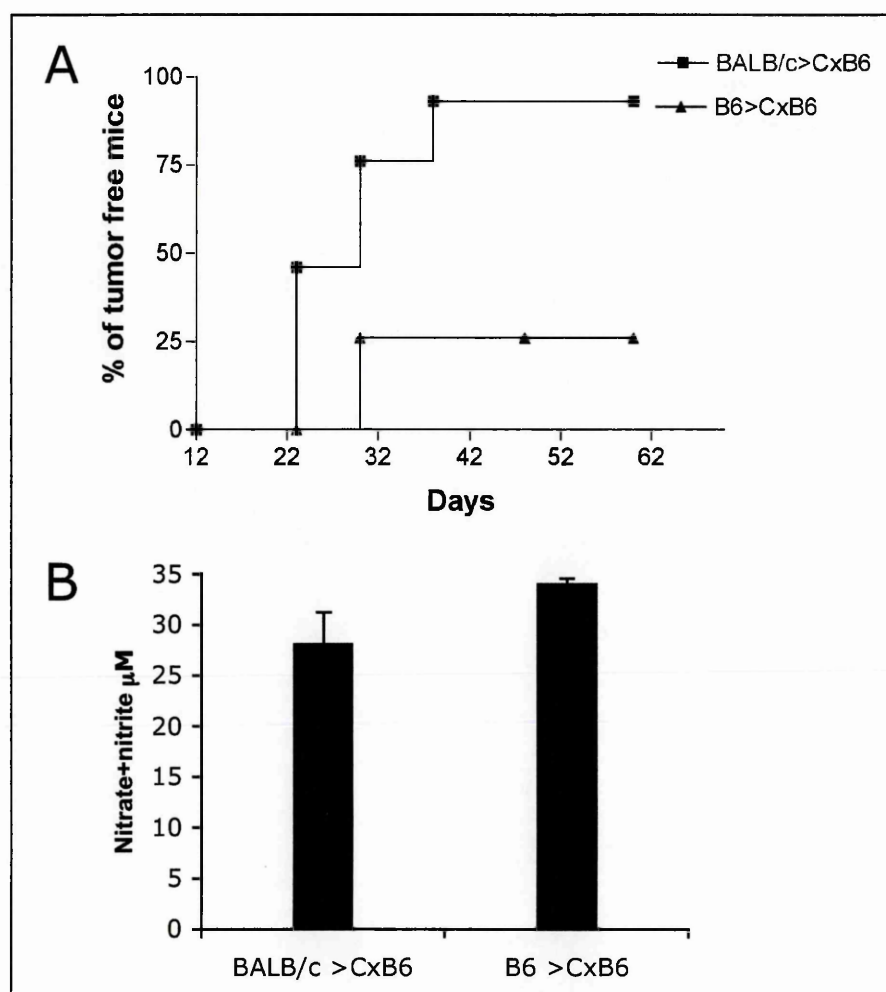


Figure 5.11 Role of tumor specific CTL in antitumor response.

A) BALB/c>CB6 and B6>CB6 chimeras were inoculated with 2×10^5 TSA cells. 12 days later, tumors were treated with AdCCL16, CpG and anti-IL10R. Cumulative data from two independent experiments, each performed with 8 mice, are shown. B) In a separate experiment, AdCCL16-treated tumors (n=5/group) were collected 4 h after CpG plus anti-IL10R treatment and processed to measure NO production (results expressed as mean \pm SD).

5.3.5 CD40 KO and IL-12 KO mice fails to reject tumors in response to CCL16, CpG plus anti-IL10R treatment

To test whether the requirement for CD4 T cell subsets reflects the need for interaction between DC and CD4 T cells (since TSA tumor is MHC class-II negative), antitumor effects of the combination treatment was evaluated in mice lacking CD40 a key molecule for the interaction between DC and CD4 T cells during CTL induction (Schoenberger et al., 1998). Moreover since CD40 cross-linking on DC by CD40L expressed by CD4 cells has been shown to induce IL-12 that is the primary cytokine bridging innate and adaptive immune responses (Trinchieri, 2003), we also tested the efficacy of the combined treatment in IL12p35-KO mice. As shown in Fig. 5.12A both CD40- and IL-12p35-KO mouse strains failed to reject TSA tumors in response to the combination protocol.

To determine whether lack of tumor rejection in CD40 and IL-12 KO mice might rest in defective T cell activation the ability of mice treated with the combined treatment to generate CTL in absence of CD40 or IL-12 molecules was compared to wt mice. As shown in Fig. 5.12 B both mouse strains failed to generate CTL.

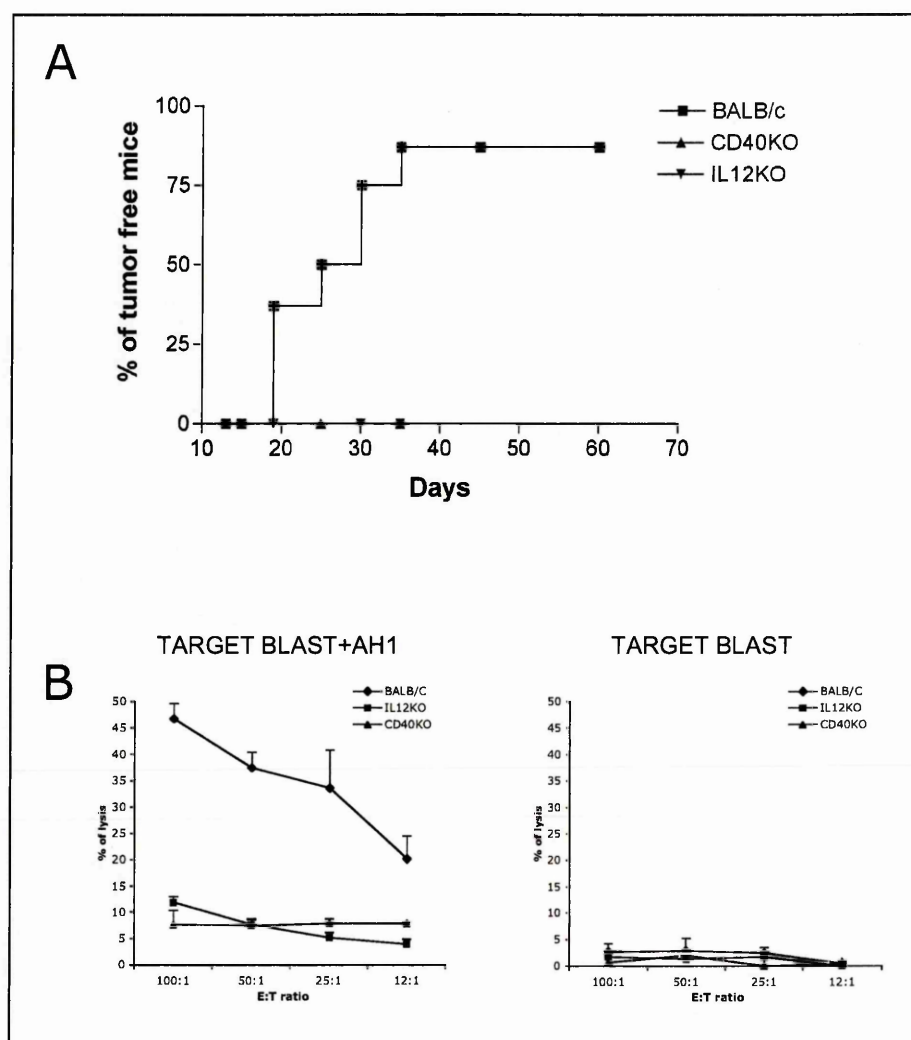


Figure 5.12 CCL16, CpG and anti-IL10R induced tumor rejection is abolished in mice deficient for CD40 or IL-12.

A) Tumor rejection was monitored in wt, CD40-KO and IL-12p35-KO mice ($n=8$). B) CTL activity was monitored 30 days after treatment, upon *in vitro* restimulation with the AH1 antigenic peptide of TSA, against blast cells loaded or not with the AH1 peptide. (mean \pm SD from three mice per group).

5.3.6 The combination of CCL16, CpG and antiIL10R restores tumor infiltrating DC functions and bridges innate and adaptive immunity.

In addition to macrophages, AdCCL16 treatment recruited DC in TSA tumors (Fig. 4A). The functions of these TIDC, however, can be impaired by the tumor milieu. The generation of CD8 tumor antigen-specific T cell response in the treated mice and their requirement for tumor eradication suggested that the combined treatment restored DC functions.

Therefore the phenotype and functions of TIDC from treated vs non-treated animals was evaluated. DC infiltrating AdCCL16-treated tumors showed an immature phenotype similar to the Addl70-3 control treated group, suggesting that the majority of DC recruited by CCL16 at tumor site are impaired in their antigen presentation functions.

AdCCL16 combined with CpG and anti-IL10R induced up-regulation of CD40, CD80 and CD86 in CD11c⁺ DC whereas the omission of anti-IL10R abrogated this effect (Fig. 5.13). Consistent with the activated phenotype, TIDC from the treated mice produced high levels of IL-12 and TNF- α (Fig. 5.14 C-D) and vigorously stimulated allogeneic naïve T cells to proliferate and produce IFN- γ (Fig. 5.14 A-B).

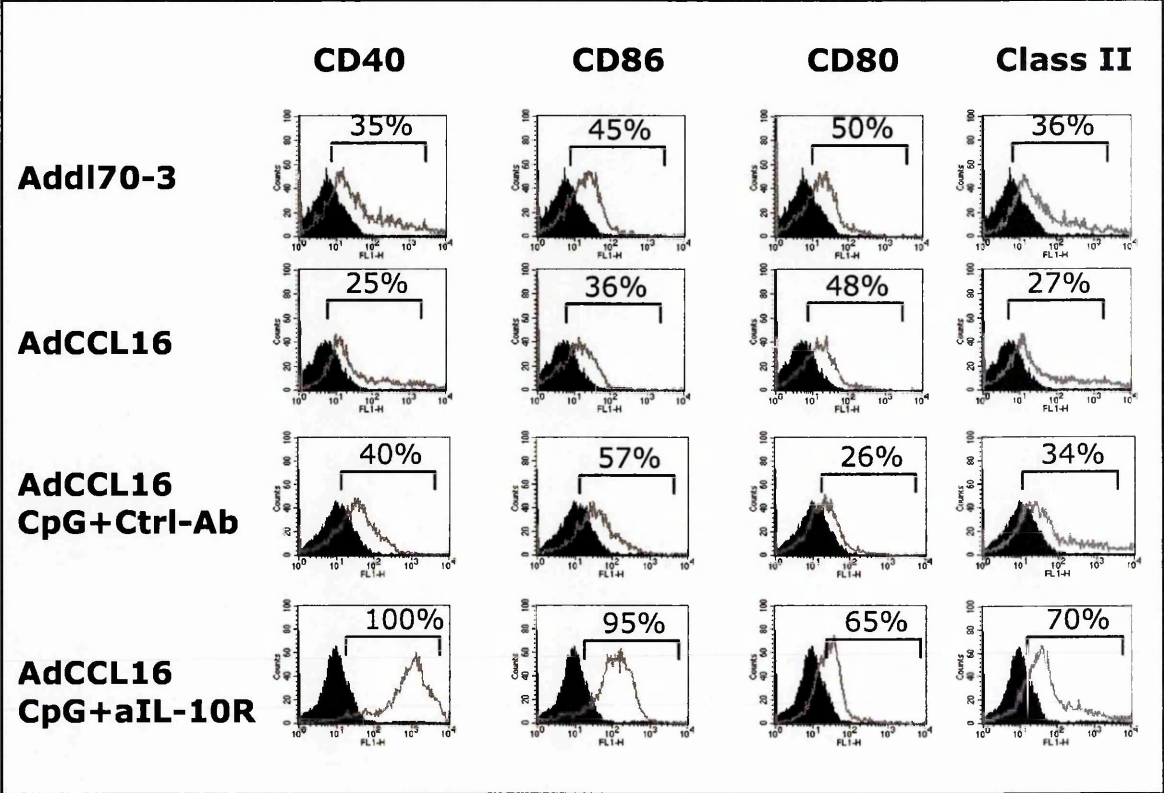


Figure 5.13 Phenotypic characterization of tumor infiltrating DC.

TSA tumor were treated with AdCCL16 or Addl70-3 and 36 h later treated with PBS or with CpG alone or combined with anti-IL10R Ab. TIDC were purified from treated tumors 10 h later last treatment and analyzed by FACS analysis for the activation markers CD40, CD80, CD86 and MHC-II.

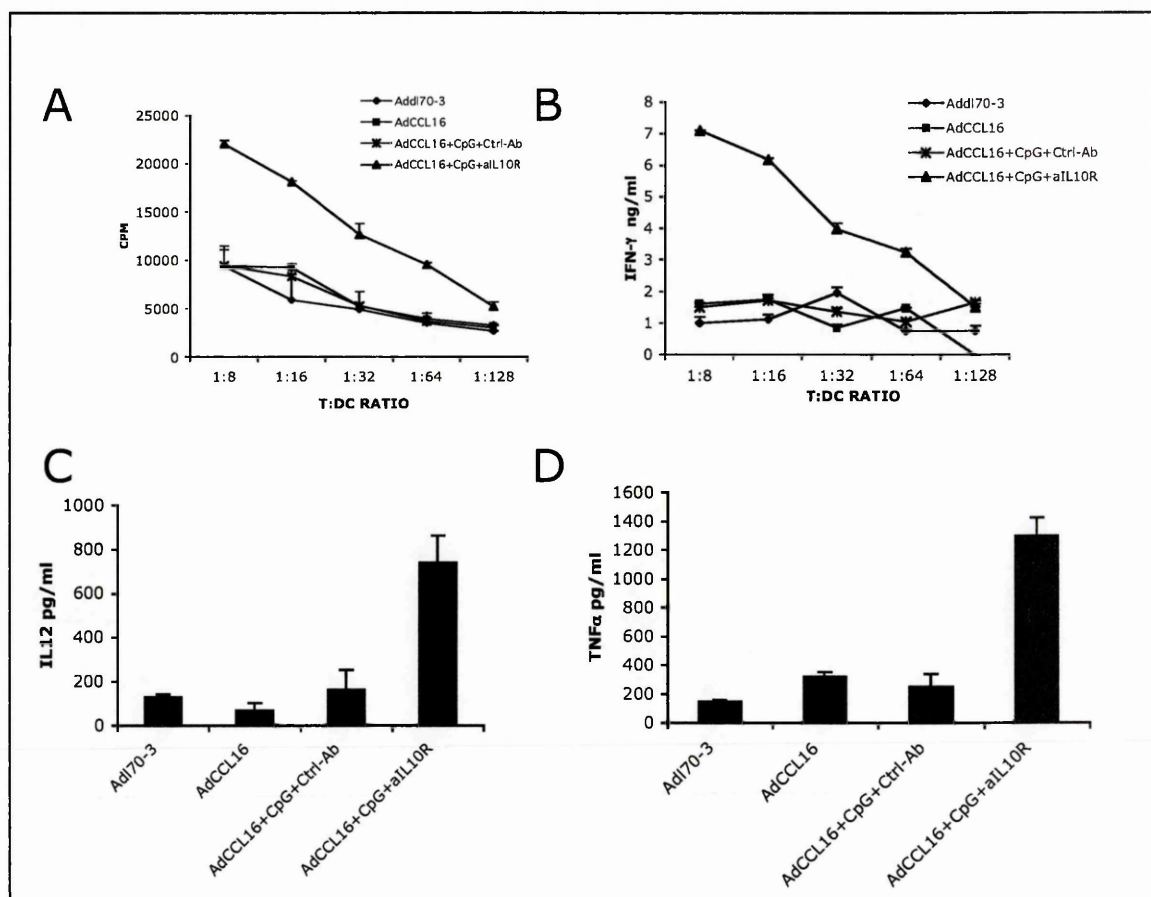


Figure 5.14 Functional characterization of tumor infiltrating DC.

TSA tumor were treated with AdCCL16 or Addl70-3 and 36 h later treated with PBS or with CpG alone or combined with anti-IL10R Ab. TIDC were purified from treated tumors 10 h later last treatment an analyzed for their ability to: stimulate proliferation (A) and IFN-γ production of allogeneic T cells in an MLR assay (B) and for IL-12 and TNF-α production by ELISA, C and D, respectively. Data from one representative experiments out of three with similar results (mean± SD of the triplicates).

In order to prime the anti-tumor CTL response observed in treated mice, either TIDC migrated to the DLN before the fast and massive necrosis of the tumor mass affected their viability or DC other than TIDC were involved in the onset of the adaptive response, for example by uptaking tumor-antigens released into lymphoid sites following tumor necrosis. To investigate these possibilities, DLN were collected 6 and 16 h after the different types of treatments from mice in which FITC-labeled beads were injected as tracer in the tumor mass. CCL16 treatment combined or not with CpG plus anti-IL10R induced the highest number of FITC-labeled DC detected in DLN collected 6 h after treatment, a number that did not significantly differ from that of DC collected after 16 h (Fig. 5.15A). This observation suggested that the majority of TIDC migrated to the DLN during the first 6 h post-treatment. Indeed, mice in which the injection of FITC-beads was delayed 6 h post-treatment showed a significant lower number of FITC-labeled DC in DLN than the mice receiving the beads together with the treatment (Fig. 5.15B).

Finally, the functional requirement of DC migration for tumor eradication was tested using CCR7-KO mice, whose DC cannot respond to CCL19 and CCL21. No more than 25% of TSA tumor bearing CCR7-KO eradicated the tumor in response to the combined treatment (Fig. 5.16).

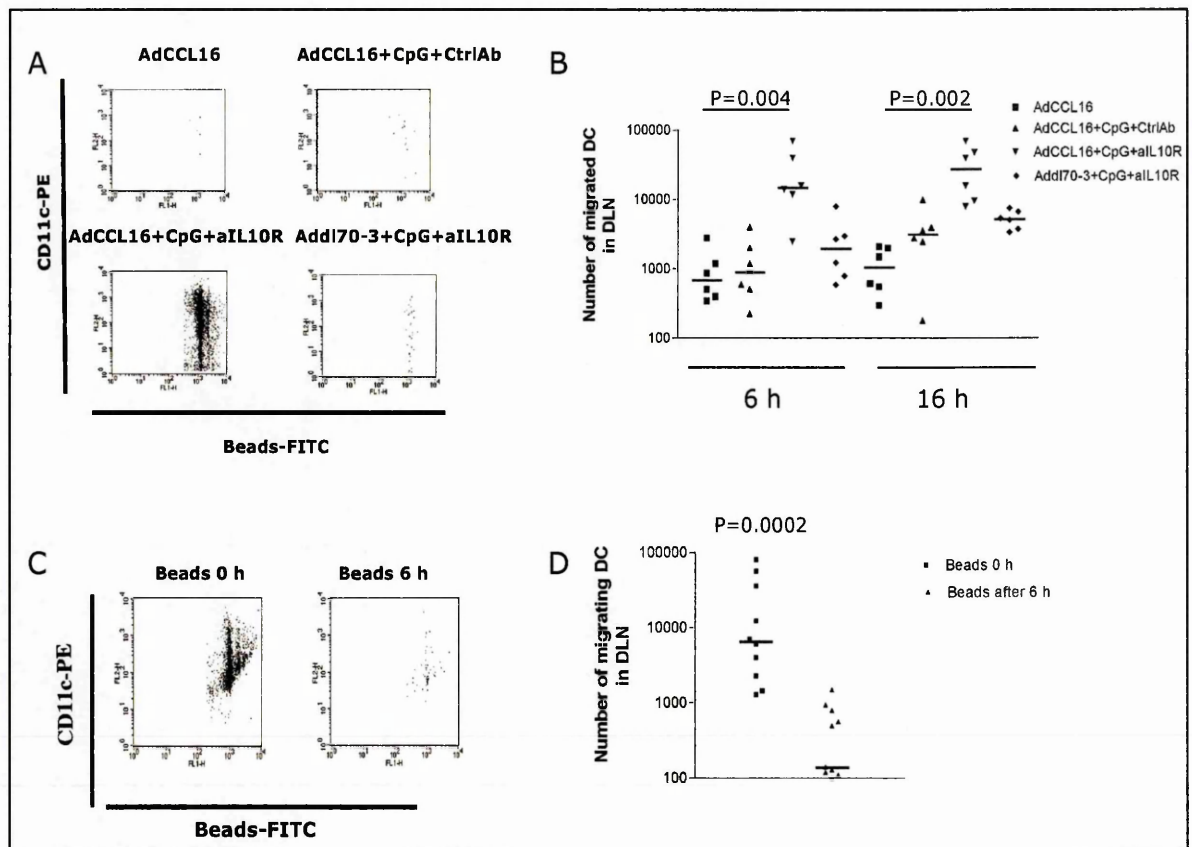


Figure 5.15 CCL16, CpG and anti-IL10R substantially increase the number of CD11c+ DC migrating to DLN.

A) TSA tumors were treated with AdCCL16 or Addl70-3 and 36 h later with CpG together with fluorescent latex beads and anti-IL10R or control Ab given systemically. 6 h or 16 h later, DLNs were analyzed for the presence of CD11c+ cells carrying FITC+ beads (representative plots on the left). Total number of migrated DC is reported (right). B) Tumors were treated with AdCCL16 and 36 h later antiIL-10R was given systemically and fluorescent beads were injected together with CpG or 6 h later. DLN were collected 16 h after beads injection (representative plots are shown on the left and cumulative data of migrated DC on the right). Each dot in cumulative graphs refers to a single DLN collected from an individual treated mouse.

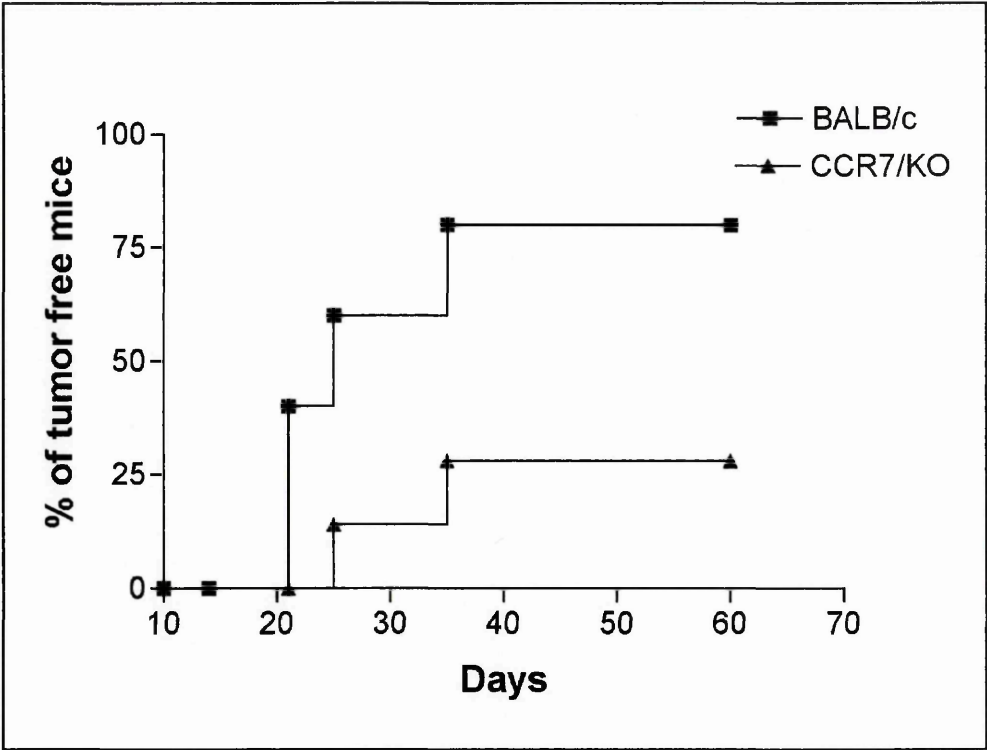


Figure 5.16 CCL16, CpG and anti-IL10R induced tumor rejection is abolished in mice deficient for CCR7.

Tumor rejection was monitored in wt and CCR7 KO (*n*=8).

5.3.7 Mice lacking TNF- α in their BM compartment are unable to develop hemorrhagic necrosis and to reject large tumors.

The results presented above showed that the combination treatment results in an early inflammatory response accountable for the initial tumor debulking and for the induction of the adaptive response, which it was demonstrated necessary to completely reject tumors. However in none of the above experiments it was demonstrated what was the impact of the innate response in the tumor rejection process. This was investigated creating chimeras mice bearing BM from TNF-KO, which lack a key factor responsible of the innate response. Chimeras were generated by transferring BM from TNF-KO mice into congenic

B6.SJL mice. The donor and recipient strains differed for the CD45 allele that allowed to follow the engraftment of TNF-KO BM after transplantation.

Moreover since TNF- α gene deletion spans nearby MHC sequence, donor cells were tolerized to host MHC, avoiding tumors rejection because of subtle MHC disparity.

Injection of MCA38 in these mice produces large tumors that if treated with the combined treatment were not rejected (Fig. 5.18A). Macroscopical examination of treated-tumors revealed that in absence of TNF- α the hemorrhagic necrosis was strongly defective (Fig. 5.17 A-B). Histological analysis showed absence of necrosis area in mice lacking TNF- α if compared with wt animals confirming the above observations (Fig. 5.17 C-D). Lack of the innate response impact the ability of TIDC to migrate to DLN as demonstrated by a strong decrease in the number of TIDC that reached DLN after treatment (Fig. 5.18 B). These results suggest that the early inflammatory response is instrumental for both tumor destruction and migration of DC to DLN.

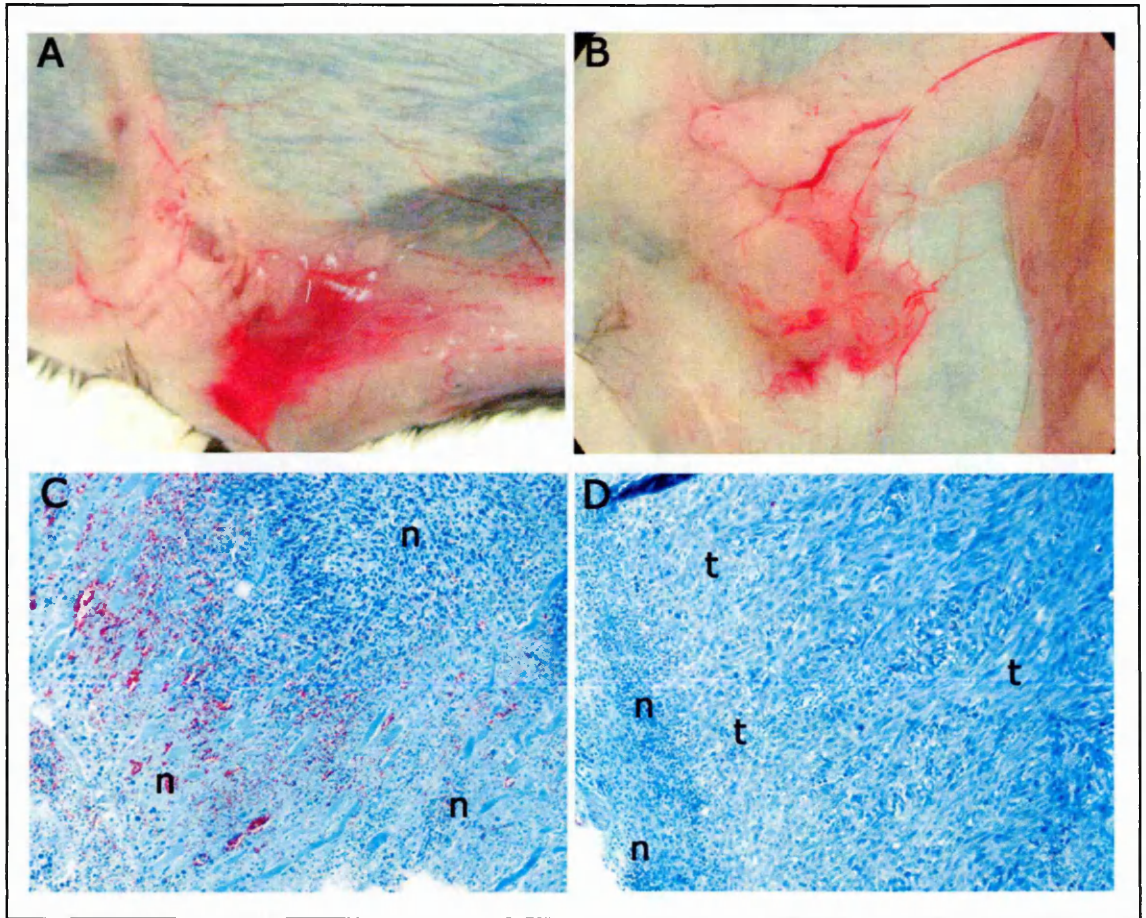


Figure 5.17 Role of TNF- α in hemorrhagic necrosis.

TNFKO> B6.SJL chimeras, differing in the CD45 allele, were used to test the role of leukocytes produced TNF- α . B6.SJL (A-C) and chimeras (B-D) were injected with MCA38 carcinoma cells and were treated, when palpable, with AdCCL16 combined with CpG plus antiIL10R. Macroscopic evaluation (A-B) trichomic staining (C-D) of tumor sections collected 16 h after treatment. Necrotic area (n) and intact tumor area (t) are indicated in the figure.

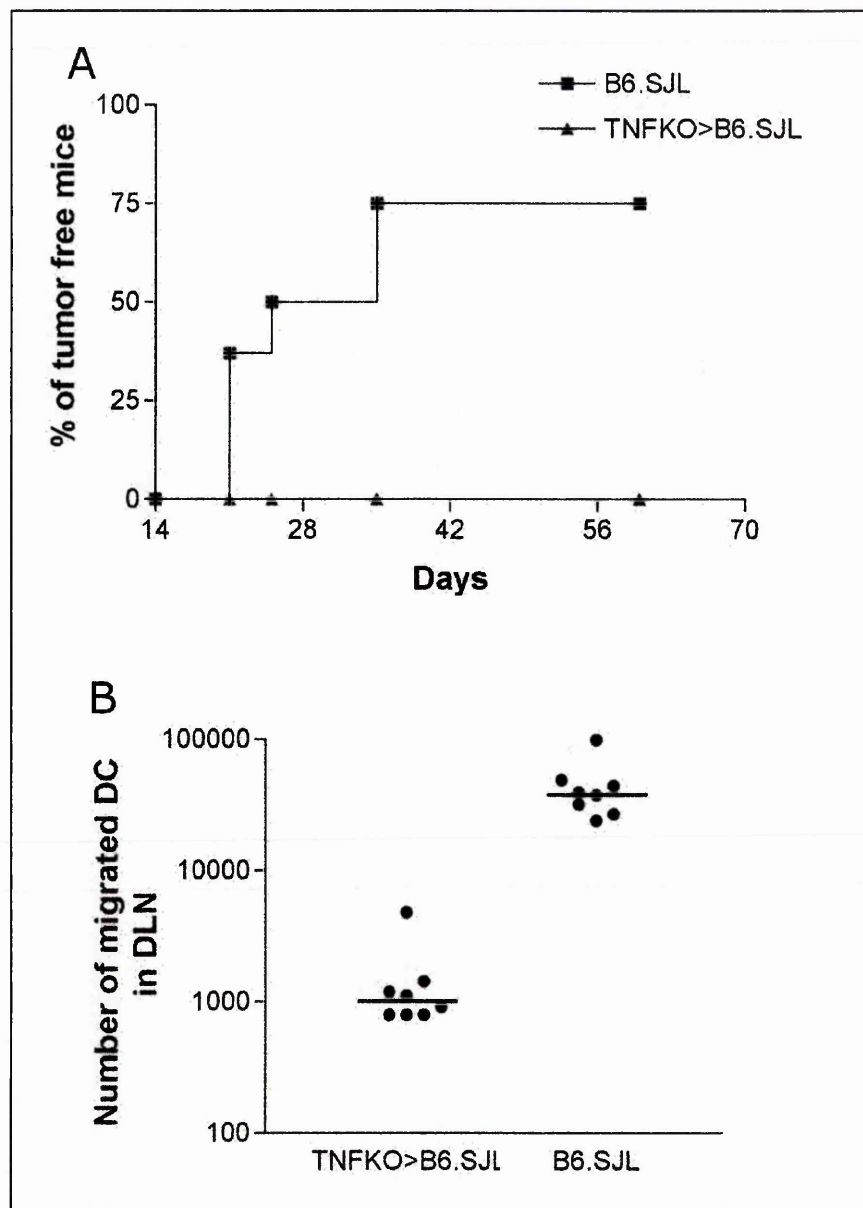


Figure 5.18 Role of the inflammatory response in tumor rejection and TIDC migration.

TNFKO> B6.SJL chimeras, defective for inflammatory response (Fig 5.17) were used to test the role of the early inflammatory response in tumor rejection and TIDC migration. A) Tumor rejection was monitored in B6.SJL wt mice and TNFKO> B6.SJL chimeras (n=8). B) MCA38 tumors were treated with AdCCL16 36 h later with CpG together with fluorescent latex beads and anti-IL10R given systemically. 10h later, DLNs were analyzed for the presence of CD11c+ cells carrying FITC+ beads. Total number of migrated DC is reported TIDC migration.

5.4 Discussion

Many studies have definitively shown that both in experimental animals and in humans, mechanisms of immune surveillance are able to control the appearance of new tumors or affect the progression of existing tumors (Dunn et al., 2004). Within the mechanisms responsible for immune surveillance, the processes of inflammation and innate resistance as well as tumor antigen specific adaptive immunity have been implicated.

Many protocols of cancer immunotherapy have been designed to induce tumor-specific CTL that are thought to be the most efficient effector cells to destroy existing tumors (Blattman and Greenberg, 2004). In patients involved in clinical trials using antigen loaded DC, CTL were often generated that were not associated with complete tumor regression probably due to a low CTL precursor frequency, tumor size and an adverse tumor microenvironment (Dunn et al., 2004; Marincola et al., 2003).

Treatment with chemokines in the attempt to recruit DC directly at the tumor site without need of in vitro manipulation has been experimentally tested in many laboratories in order to facilitate the in vivo encounter of DC with tumor antigens. As reviewed in introduction (chapter 1 section 1.3), inflammatory chemokines act on a heterogeneous population of circulating monocytes and DC that, after entering tumors, may differentiate not only into TIDC but also into tumor-associated macrophages (TAM). TAM remain in the invaded tissue while DC maintain the ability to migrate into the DLN (Imhof and Aurrand-Lions, 2004). TIDC and TAM are components of the tumor stroma and the functions of the cells recruited through chemokine manipulation are likely to be modulated by the tumor microenvironment (see chapter 1 section 1.5 and (Coussens and Werb, 2002)).

TAM are the main source of intratumor IL-10, a potent immunomodulating molecule able to turn down both innate and immune responses by affecting DC functions and acting in an autocrine fashion by maintaining the infiltrating macrophages in an M2 state (Moore et al., 2001). In such a hostile tumor environment and in the absence of proper inflammation,

recruited DC may present antigen in an inappropriate T cell context, generating tolerance to TAA instead of immunity (Steinman et al., 2003). Strategies able to overcome such tumor escape mechanisms are the key to effective immunotherapy. Although various soluble and cellular factors have been implicated in tumor-induced immune suppression, it was previously demonstrated that IL-10 has a sufficient and essential role in determining the anergic state of TIDC. Indeed, DC infiltrating a number of different mouse tumors were shown to be refractory to maturation stimuli, unable to produce IL-12, and to stimulate T lymphocytes, unless the inhibitory effect of IL-10 was blocked using repeated treatments with an anti-IL-10R antibody (Vicari et al., 2002).

In this part of the study, we tested whether the treatment of tumor bearing mice with a chemokine able to enhance the tumor infiltration by macrophages and DC as well as to synergize with a treatment that simultaneously fights DC paralysis and subverts the macrophages phenotype from M2 to M1. In chapter 4 it was demonstrated that treatment with an adenoviral vector carrying CCL16 reduced tumor growth but induced complete tumor eradication only in a small proportion of animals. In this part of the study it was demonstrated that macrophages recruited by CCL16 produced IL-10 but not IL-12 or TNF- α , consistent with a M2 phenotype that also characterizes the resident TAM (Fig. 5.5 and 5.6). This contrasts with in vitro results showing that recombinant CCL16 effectively induced peritoneal macrophages to produce pro-inflammatory cytokines such as CCL2, IL-12 and TNF- α and to kill TSA tumor cells (Cappello et al., 2004). The in vivo results presented here indicate that CCL16-recruited macrophages became functionally inhibited by the tumor unless CpG and anti-IL10R are added to the treatment. Mice treated with this combination protocol successfully rejected established TSA, MCA38 and 4T1 tumor nodules (Fig. 5.1). When combined with CpG plus anti-IL10R, not only 60% of CCL16-treated animals rejected the 4T1 primary tumors but they were also cleared of distant metastases, whereas, as reported in chapter 4, AdCCL16 given alone, although unable to

unable to eradicate the transplanted 4T1 tumors, was effective in curing their distant metastases only when associated with the surgical excision of primary nodules, consistent with the hypothesis that the tumor is the source of both local and systemic immunosuppression.

The TLR9 agonist CpG has been widely used as adjuvant of tumor-specific antigen vaccines, or given intratumorally in a therapeutic setting (Klinman, 2004). Peritumoral injection of CpG as single agent induced tumor regression in some tumor models (Heckelsmiller et al., 2002; Kwarada et al., 2001) while has proven ineffective in others (Furumoto et al., 2004; Garbi et al., 2004). In all these reports, CpG was given repeatedly at high dose (50-100 µg/dose) to animals bearing small tumors.

In combination with CCL16 and an antibody blocking IL10R, a single CpG administration of 5 µg is sufficient to eradicate large vascularized tumors (Fig. 5.1). Considering that CpG is entering clinical trials for different applications including cancer immunotherapy and that repetitive high dose have been recently described to be toxic and to induce immunosuppression (Heikenwalder et al., 2004; Klinman, 2004) the findings reported here provide proof of concept that combination protocol can reduce both amount and frequency of CpG administrations while maintaining effectiveness. In comparison to the previous work with CpG and anti-IL10R alone (Vicari et al., 2002), the addition of CCL16 allowed to successfully treat tumors at least twice in size than those previously tested using a single rather than three injections.

One of the most impressive observations in the present study was the rapid triggering of events leading to tumor necrosis and systemic immunity. The massive hemorrhagic tumor necrosis was comparable to a localized Shwartzman reaction (Brozna, 1990), completed in 16 h, and accompanied by the release of nitric oxide (Fig. 5.3 and 5.7). Macrophages recruited by CCL16 produced IL-12 and TNF- α as soon as 6 h after CpG plus anti-IL10R treatment (Fig. 5.7) and were likely the major inflammatory cell type responsible for the

induction of tumor necrosis. Indeed, polymorphnuclear leukocytes, although frequently described to take part in necrosis and capable of tumor toxicity via production of the oxidant HOCl (Di Carlo et al., 2001), were observed to be localized at the edge of tumors treated with the combinatory regimen (Fig. 5.6), thus they are unlikely to take part in the process of tumor destruction.

The extensive hemorrhagic necrosis observed points to a central role of TNF- α and other pro-inflammatory cytokines and the participation of innate resistance mechanisms in tumor destruction. Indeed in tumor-bearing chimeric mice reconstituted with BM from TNF-KO mice, the combination treatment failed to induce hemorrhagic necrosis and to provoke tumor debulking (Fig. 5.17 and 5.18).

These results demonstrate that subversion of M2 phenotype requires that the inflammatory stimulus be combined with block of the IL-10 pathway. This is the first report showing in vivo such a rapid switch from M2 to M1 phenotype.

Tumors treated with CCL16 were also enriched in TIDC that showed the typical immature myeloid phenotype of the resident TIDC in the absence of added chemokines ((Vicari et al., 2002)) that is characterized by low lymphocyte stimulatory activity and low ability to produce inflammatory cytokines like TNF- α and IL-12 (Fig. 5.13 and 5.14). The refractory state of TIDC, together with the observed M2 phenotype of infiltrating macrophages, correlates with the poor rejection of tumors in animals treated with AdCCL16 alone (Chapter 4 Table 4.1). Along with macrophages, DC also shifted phenotypically and functionally in response to the combinatory treatment. TIDC up-regulated costimulatory molecules, produced IL-12 and TNF- α and stimulated T lymphocytes proliferation and IFN- γ secretion (Fig. 5.13 and 5.14). However, the rapid hemorrhagic necrosis induced by the treatment was so massive that it should have been expected to destroy infiltrating leukocytes along with tumor cells, questioning whether TIDC could reach the DLN before their viability was affected. In vivo experiments with fluorescent beads demonstrated that

TIDC massively migrated within the first 6 h post-treatment (Fig. 5.15). This latter result, together with the fact that significantly more DC were recovered from the DLN of mice treated with CpG and anti-IL-10R in combination with AdCCL16 than with Add170-3, indicates that the FITC-labeled DC observed in the DLN had captured latex beads within the tumor, and not within the lymph node (Fig. 5.15).

The impact of the inflammatory response on DC mobilization from the tumor was confirmed in chimeric mice reconstituted with BM from TNF-KO mice that showed both reduced inflammation and DC migration to the DLN (Fig. 5.17 and 5.18).

The more likely hypothesis is that the rapid migration from the tumor to the DLN of mature DC, at least some of which loaded with TAA, is instrumental to activate systemic immune responses and generation of CTL, before the progressive necrosis destroys intratumoral DC or prevent their ability to migrate to the DLN. This hypothesis is supported by the data of experiments shown in this chapter that analyzed separately innate and systemic response and showed that the innate response, while responsible for the early tumor necrosis, is in most mice insufficient for tumor eradication. Most of tumor-bearing CCR7-deficient mice failed to completely reject tumors when treated with CCL16 and CpG plus anti-IL10R (Fig. 5.16). CCR7, a chemokine receptor critically required for DC entering the lymphatic vessels and migration into the DLN, is upregulated in mature DC (Forster et al., 1999; Gunn et al., 1999). In addition, CCR7-KO mice have also altered homing of naïve T cells and recirculation of effectors T cells (Forster et al., 1999). Only 25% of treated CCR7-KO mice rejected tumors, a fraction similar to that of observed with other mice deprived of adaptive response, such as RAG-KO mice or mice depleted of T cells by antibody treatment (Fig. 5.10). None of the treated CCR7-KO that have eradicated the primary tumor had the ability to reject a challenge with live tumor cells, demonstrating that in these mice only the innate response was operative.

Accordingly, in B6>CXB6 chimera mice in which B6-induced CTL cannot recognize TSA (H-2^d) tumors, the observed low tumor rejection rate was due to the innate response (Fig. 5.11). A more detailed analysis of the role of CD4 and CD8 T cells indicated that, while depletion of CD8 T lymphocytes either during the priming or the effector phase reduce anti-tumor efficacy, CD4 T cells are needed for efficient priming (Fig.5.10). CD4 T cell help for the induction of effector CD8 T cells is mediated by CD40/CD40L interaction with DC (Mackey et al., 1998a). Accordingly, lack of tumor rejection and defective CTL induction was observed in mice lacking CD40 indicating the need of a correct DC-T cell cross-talk (Fig. 5.12).

IL-12 bridges innate and adaptive immunity by promoting CTL induction, NK function and Th1 differentiation of CD4 helper T cells (Trinchieri, 2003). Impaired anti-tumor response in the absence of CD40/CD40L interaction might result from a defective IL-12 production (Mackey et al., 1998b). Indeed abrogation of both tumor rejection and CTL generation was observed in mice lacking IL-12 (Fig. 5.12). Although alternative pathways of CTL induction independent from CD40 and IL-12 have been described, they were mainly effective against non-tolerized antigens and/or in the presence of high number of specific T cells precursors (Lu et al., 2000; Wan et al., 2001) and thus unlikely to be effective in an anti-tumor response. Indeed, in this chapter it was demonstrated that to induce adaptive immunity to TAA the presence of high inflammatory stimuli cannot compensate for the lack of CD40/CD40L co-stimulation that is probably required for full maturation and activation of DC in the lymph nodes, including optimal production of IL-12 following T cell encounter (Schulz et al., 2000).

Taken together, the results presented in this chapter strongly support that the manipulation of macrophages and DC recruitment within tumors together with their activation and relief of tumor immunosuppression may represent a potentially effective anti-tumor therapy.

CHAPTER 6: SUMMARY AND FUTURE PLANS

6.1 Summary

Aim of the first part of this thesis was to characterize the antitumor activity of the neglected human chemokine, CCL16. Delivery of CCL16 by an adenoviral vector into prestablished murine recruited T cells, macrophages and DC at the tumor site and led to rapid swelling of draining lymph nodes with accumulation of DC in the T cell area. However, despite the local accumulation of reactive cell infiltrate and the induction of a systemic immune response, large primary tumors were not completely rejected. While the result might have been expected considering the difficulties of rejecting well-established, vascularized tumors, it pointed to the utility of intralesional injection before surgery, for prevention of metastases in the presence of bad prognostic factors. Experimentally, this was investigated using the spontaneously metastatic 4T1 mammary carcinoma, which spread to several distant organs when priming tumors were surgically excised at the size of 2mm. Injection of AdCCL16 5 days before surgery results in mice either free or with less than 10 metastases in the lung whereas all mice, treated with empty adenovector or untreated, have hundreds of metastases.

In the second part of the project the tumor escape mechanisms responsible for the paralysis of CCL16 induced leukocytes infiltration were characterized. Macrophages infiltrating CCL16-treated as well as untreated tumors showed an M2 phenotype characterized by secretion of IL-10 but not of IL-12 or TNF- α production and by lack of cytotoxicity against the tumor. Similarly, TIDC purified from CCL16-treated tumors, showed an immature phenotype suggesting that their migration to DLN might not only result in an inefficient T cell priming, rather in induction of tolerance to TAA. These findings are in line with what found by many groups reporting that macrophages and DC, spontaneously infiltrating human and murine tumors, are functionally paralyzed or working in favour of tumor

progression. This suggests a cautionary note for those clinical approaches involving enrichment of immune cells at the tumor site since they might be rendered ineffective by the strong tumor escape mechanisms that co-opt recruited cells to act pro-tumorally.

The combination of CCL16 and CpG given locally together with the systemic administration of a mAb to IL10R induced, within a few hours, a shift of resident and recruited tumor-infiltrating macrophages from the M2 to M1 type and cured the majority of mice bearing large tumors. In addition, TIDC rapidly up-regulated costimulatory molecules and secreted inflammatory cytokines such as IL-12 and TNF- α . This potent inflammatory response not only induced massive hemorrhagic necrosis and tumor shrinkage but also a fast DC migration to DLN preceding tumor necrosis and allowing the generation of tumor-specific CTL response able to clear any tumor remnant.

Innate and adaptive responses work in concert to reject large tumors. Different experimental approaches confirmed that the innate response is responsible of initial tumor debulking while capable to reject completely the tumors in a fraction of 30% of treated mice. The importance of the early inflammatory response was underscored by experiments showing that in absence of TNF- α , tumors were not rejected and TIDC remained subjected to tumor induced suppression.

In conclusion the work presented here revealed the mechanisms by which large numbers of tumor-infiltrating macrophages and dendritic cells can be redirected to become potent effectors and activators of the innate and adaptive immunity. Overcoming the strong local immunosuppression was shown to be the key to reject successfully large tumors and suggest a new strategy for clinical interventions.

6.1.1 Future plans

The following points provide a framework for future studies.

6.1.2 Role of CCL2 in AdCCL16 antitumor activity.

In this thesis it was shown that CCL16 treated tumors are infiltrated by macrophages and DC. These subsets of cells become activated after CpG plus antiIL10R treatment to become potent effectors of the innate response. CCL16 potentially enhance the release of CCL2 from macrophages (Cappello et al., 2004), DC and T cells (Chapter 3). CCL2 is one of the most active chemokine attracting mononuclear cells. This property has led to the suggestion that CCL2 could have antitumor properties in vivo. Experiments in animal models demonstrated that high level of CCL2 expression of at the tumor site can suppress tumor growth by recruiting macrophages and DC in high number (Conti and Rollins, 2004). Therefore, it is possible that parts of the antitumor properties exerted by local delivery of CCL16 are mediated through CCL2. To this extent AdCCL16 treatment alone could be performed in CCL2 KO mice to evaluate number of infiltrating mononuclear cells and their M2 phenotype.

6.1.3 Dissecting the role of IL-12 in tumor rejection mediated by AdCCL16 and CpG plus antiIL10R treatment.

IL-12 is a cytokine playing essential role both innate and adaptive immunity. IL-12 enhances the generation of cytotoxic T lymphocytes and augments their cytotoxic activity through upregulation of perforin and granzyme. IL-12 secretion by DC following cognate interaction with Th cells is dependent from CD40/CD40L interaction (Colombo and Trinchieri, 2002; Trinchieri, 2003).

In chapter 5 it has been demonstrated that mice deficient of CD40 or IL-12 were unable to reject TSA tumors treated with AdCCL16, CpG and anti-IL10R, as well as to generate tumor specific CTL. Together with its role in modulating T cell response IL-12 has also been implicated in sustaining the innate response. Products from microorganism such as double strand RNA, bacterial DNA and CpG containing sequences are strong inducers of IL-12 production by macrophages and DC. Indeed, both TIDC and macrophages recruited by CCL16 at tumor site produce IL-12 after CpG administration and block of IL10R. Macrophages from the IL-12KO mice, could not properly respond to CpG triggering, and might provide an insufficient inflammatory response with consequences on both the innate and adaptive responses. This hypothesis could be tested as follows:

- TSA tumors grown in wt and IL-12KO mice treated with the AdCCL16, CpG and anti-IL10R could be evaluated for local innate response. Histological analysis could be used to evaluate the extension of necrotic destruction and production of TNF- α and nitric oxide. Moreover tumor-infiltrating macrophages collected after the treatments performed in IL-12KO and wt animals could be evaluated in vitro for their ability to lyse TSA tumor cells.

6.1.4 Alternative TLR for the activation of the tumor infiltrate

In the present studies in mice, it was used human CCL16 to promote macrophage and DC recruitment within mouse tumors, since its mouse homologue exists only as a pseudogene (Shoudai et al., 1998; Youn et al., 1998), thus facilitating the possibility of translation of the therapeutic protocol into the clinical setting. However, the fact that different subsets of cells respond to CpG in humans and mice raises the question of whether CpG would induce similar response in human tumors. In humans, plasmacytoid DC (pDC) are the

main cells triggered by CpG that, in the mouse, activates indistinctly myeloid DC, monocytes/macrophages and pDC.

Human blood pDC can differentiate into fully competent DC following microbial stimuli such as CpG (Kadowaki et al., 2001) and abundant pDC infiltration was observed in human ovarian carcinoma and melanoma (Salio et al., 2003; Vermi et al., 2003; Zou et al., 2001). Although the exact role of pDC infiltrating human tumors is yet to be determined, the observation of their immature phenotype and their inability to release IFN- α suggest that infiltrating pDC are negatively regulated by the tumor microenvironment. Indeed, in vitro, blocking of IL10R re-established the ability of pDC extracted from tumors to stimulate T lymphocytes (Zou et al., 2001).

Although injection of CpG in human tumors would not directly activate macrophages or myeloid DC, through induction of IFN- α by pDC CpG could indirectly activate them leading to a cascade of events similar to what has been described here for the mouse system.

Alternatively, other ligand of Toll-like receptors that activate both macrophages and DC in humans could replace CpG for the treatment of human cancer.

Bacillus Calmette-Guérin cell-wall skeleton (BCG-CWS), which has been used as a potent adjuvant therapy in patients with cancer, has been identified to signal through TLR2 and TLR4 in human (Tsuji et al., 2000). Indeed, the costimulatory molecules CD80/CD83/CD86 and inflammatory cytokines TNF- α and IL-12 are up-regulated in human DCs after stimulation with BCG-CWS in a TLR2/4 dependent manner (Tsuji et al., 2000). A clinical trial where more than 600 patients were treated following postoperative surgery of bladder cancer brought good prognosis for more than 60% of the treated patients (Hayashi, 1998). One of the notable phenomena after BCG bladder instillation in human patients is a transient secretion of several inflammatory cytokines such as INF-g, IL-12 and IL-2 in BCG-CWS responders. On the contrary patients that fails to respond to

BCG-CWS therapy showed high levels of IL-10 (Elsasser-Beile et al., 2000; O'Donnell, 1996). Similarly, in mouse models IFN- γ and IL-12 but not IL-10 and IL-4 have been suggested to be required for the antitumor activity against an orthotopic model of bladder cancer (Riemensberger et al., 2002).

Waiting for recombinant molecules triggering TLR2 and TLR4 BCG-CWS, although contaminated of bacterial product, can be tested to substitute CpG to activate both human DC and macrophages infiltrating tumors. To test this hypothesis the following assays could be performed.

- test the antitumor activity of CCL16 combined with intratumoral injection of BGC and systemic block of IL10R on prestablished TSA tumors.
- characterize the innate response by immunoistochemistry.
- characterize the activation status of TIDC and TAM following BCG treatment.
- characterize the migratory capacity of TIDC activated by BCG treatment.

PUBLICATIONS

7.1 Publications on the thesis project

Guiducci C, Di Carlo E, Parenza M, Hitt M, Giovarelli M, Musiani P and Colombo MP. Intralesional injection of adenovirus encoding CC chemokine ligand 16 inhibits mammary tumor growth and prevents metastatic-induced death after surgical removal of the treated primary tumor. *J Immunol.* 2004 Apr 1;172(7):4026-36.

Guiducci C, Vicari AP., Sangaletti S., Trinchieri G. and Colombo MP. Redirecting in vivo elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. In press *Cancer Research*.

7.2 Other publications during the PhD period

Guiducci C*, Valzasina B*, Dislich H and Colombo MP. CD40/CD40L interaction regulates CD4+CD25+ T reg homeostasis likely through DC produced IL-2. *Eur J Immunol.* 2005 Feb;35(2):557-67.

Valzasina B*, **Guiducci C***, Dislich H, Weinberg AD and Colombo MP. Direct signalling of OX40 and GITR on CD4+CD25+ regulatory T cells breaks their suppression function. In press *Blood*. * *BV and CG contributed equally to the above 2 papers*

Sangaletti S, Stoppacciaro A, **Guiducci C**, Torrisi MR and Colombo MP. Leukocyte, rather than tumor-produced SPARC, determines stroma and collagen type IV deposition in mammary carcinoma. *J Exp Med.* 2003 Nov 17;198(10):1475-85

Massa C, **Guiducci C**, Arioli I, Parenza M, Colombo MP and Melani C. Enhanced efficacy of tumor cell vaccines transfected with secretable hsp70. *Cancer Res.* 2004 Feb 15;64(4):1502-8.

Dalerba P., **Guiducci C.**, Poliani P.L., Cifola I., Parenza M., Frattini M., Gallino G. Carnevali I. Andreola S., Di Giulio I., Lombardo C., Rivoltini L., Schweighoffer T., Belli F., Colombo M.P., Parmiani G. and Castelli C. Reconstitution of hTERT expression rescues metastatic colorectal carcinoma primary cultures from *in vitro* senescence: evidence against immortality as a constitutive trait of tumor cells. In press, *Cancer Research*.

Sangaletti S, Gioiosa L, **Guiducci C**, Rotta G, Rescigno M, Stoppacciaro A, Chiodoni C and Colombo MP. Accelerated dendritic cell migration and contact hypersensitivity in absence of the matricellular protein SPARC. Submitted, Journal of Cell Science.

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